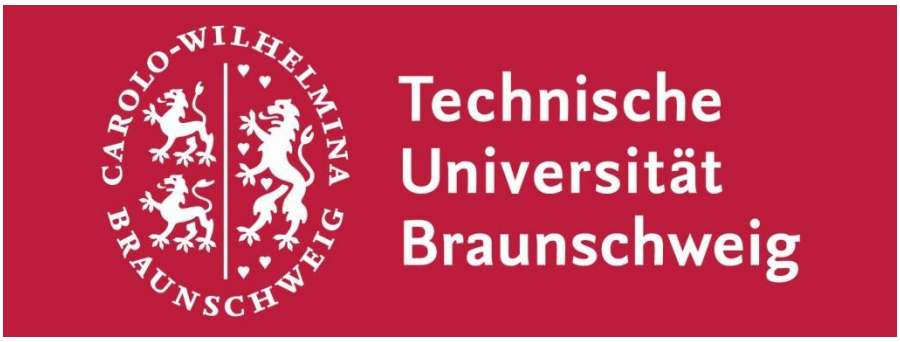

Tailoring T helper 17 induction following vaccination by antigen dosage and adjuvant usage

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

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D i s s e r t a t i o n

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Abbreviations

αGCPEG	pegylated alpha galactosylceramide
AA	amino acid
Ab	antibody
Ag	antigen
AhR	aryl hydrocarbon receptor
APC	allophycocyanin or antigen-presenting cell
APL	altered peptide ligand
c-di-AMP	bis(3',5')-cyclic dimeric adenosine monophosphate
c-di-GMP	bis(3',5')-cyclic dimeric guanosine monophosphate
ATRA	*all*-trans retinoic acid
BD	Becton, Dickinson and Company AG
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
BPPcysPEG	S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl polyethylene glycol
CD	cluster of differentiation
CFA	Complete Freund's Adjuvant
Ci	Curie
Con A	concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
Cy5/7	Cyanine 5/7
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double-stranded
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
eBio	eBioscience AG
e.g.	*exempli gratia*
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot Assay

ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanate
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony stimulating factor
gp	glycoprotein
HA	hemagglutinin
h.i.	heat inactivated
h	hours
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
ICOS	inducible T cell co-stimulator
i.e.	*id est*
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
i.n.	intranasal
i.p.	intraperitoneal
KO	knock-out
LN	lymph node
M	molar
MACS	magnetic-activated cell sorting
MALP-2	macrophage activating lipopeptide of 2 kDa
MFI	median fluorescence intensity
MHC	major histocompatibility complex
min	minute
mM	milli molar
MS	multiple sclerosis
NA	neuraminidase
NKT cell	natural killer T cell
OVA	ovalbumin
OVA-pep	ovalbumin peptide

PAMP	pathogen-associated molecular pattern
PB	Pacific Blue
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PEG	polyethylene glycol
PRR	pattern-recognition receptor
RA	rheumatoid arthritis
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SN	supernatant
SSC	side scatter
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF	transforming growth factor
Th1/2/17	T helper cells 1/2/17
TLR	Toll-like receptor
TNF	tumor necrosis factor

1 Abstract

The immune system consists of two main components. The first is the innate immune system, which acts as the first line of defense in a rapid and unspecific manner. The second is the adaptive immune system, which is based on receptor rearrangement after the first encounter with a pathogen. It is responsible for a strong, sophisticated, highly-specific and long-lasting response. These responses encompass pathogen specific antibodies (Abs) and cytotoxic T cells. This complex system of adaptive immunity is orchestrated by specialized $CD4^+$ T cells, the so-called T helper (Th) cells. These cells were classically divided into two major subsets by their functions. The Th1 cells that are mainly responsible for defense against intracellular pathogens, and the Th2 cells that are needed to initiate efficient production of Abs against extracellular pathogens and helminths. Only a few years ago a third subset named Th17 was discovered, which is characterized by the production of the cytokine interleukin-17 (IL-17). This subset is known to be important for optimal clearance of certain pathogens, but can also contribute to autoimmune and inflammatory diseases. However, up to now there is fragmentary knowledge about Th17 cells, especially regarding their induction following infection and vaccination. Depending on the specific pathogen, the appropriate Th phenotype should be stimulated in order to trigger optimal protective immunity against infection. Therefore, it is essential to elucidate under which circumstances a particular Th cell type is induced (*e.g.* after vaccination). Previous studies suggest that antigen (Ag) dose plays an important role in Th1/Th2 polarization.

In this work, *in vitro* and *in vivo* studies using wild type and transgenic mouse models were performed to get insights into the mechanisms of Th cell induction, particularly in relation to the Ag dose. *In vitro* proliferation assays comparing antigen dose dependent Th polarization in cells from DO11.10 and OTII T cell receptor (TCR) transgenic mice (all TCRs in these animals are specific for ovalbumin [OVA]) revealed striking differences in Th17 induction between the two mouse strains. In contrast, no differences were detected with respect to Th1 and Th2 polarization. DO11.10 cells show an induction of Th17 cells at low and high Ag doses, whereas OTII cells polarize to Th17 cells exclusively in the presence of high Ag doses. Experiments with fluorescent labeled Ag identified differences in TCR affinity to the

Ag as one reason for the observed phenomenon. *In vivo* immunizations of wild type mice with the model Ag OVA led to Th17 induction at low Ag doses. Recent studies provided evidence that Th17 cells are important in fighting influenza infections. Inspired from these results, immunizations with H5N1 virosomes followed by an H5N1 viral challenge were performed to investigate whether low doses of H5N1 virosomes lead to increased Th17 immune responses and improved protection as compared to standard Ag dose immunizations. To extensively monitor Th responses in vaccinated animals a sophisticated 9 color staining panel for flow cytometry analysis was established, which also allowed assessment of the induction of polyfunctional T cells. The results revealed protection of mice immunized with low virosome dosage after challenge with the influenza virus. However, the number of Th17 cells was not increased in these animals, compared to those receiving higher doses.

Since Th17 immune responses are not always beneficial for the host, as a secondary aim of this work activities were focused on the establishment of strategies to block Th17 induction at will. To this end, the specific blocking of Th17 induction by a pegylated derivate of α-galactosylceramide (αGCPEG) was investigated and dissected. *In vitro* and *in vivo* experiments demonstrated that natural killer T (NKT) cells released high amounts of interleukin (IL)-4 and interferon (IFN)γ post αGCPEG stimulation and subsequently blocked the induction of Th 17 cells.

Taken together the results of this thesis contribute to a better understanding of Th biology, especially with respect to Th17 induction and blockage. Knowledge about Th polarization is a key for the development of tailored immune responses following vaccinations. It is expected that the tools and strategies established during this work will facilitate the development of vaccines able to stimulate tailored Th17 responses to promote optimal protection against infection.

2 Introduction

2.1 The immune system

Every higher organism faces daily potential harmful pathogens. During evolution a number of defense mechanisms evolved, which are able to protect the host from these pathogens. In vertebrates the first line of defense are physical barriers, like the skin or mucosa with its antibacterial peptides, fatty acids, enzymes and low pH value which hinder microbial pathogens to enter the body. In addition, the immune system (a network of specialized cells, molecules, tissues and organs) controls the pathogens that have penetrated body barriers. The immune system can be subdivided into the innate and the adaptive immune systems, which have different cellular components and functions (Fig. 1) [1, 2].

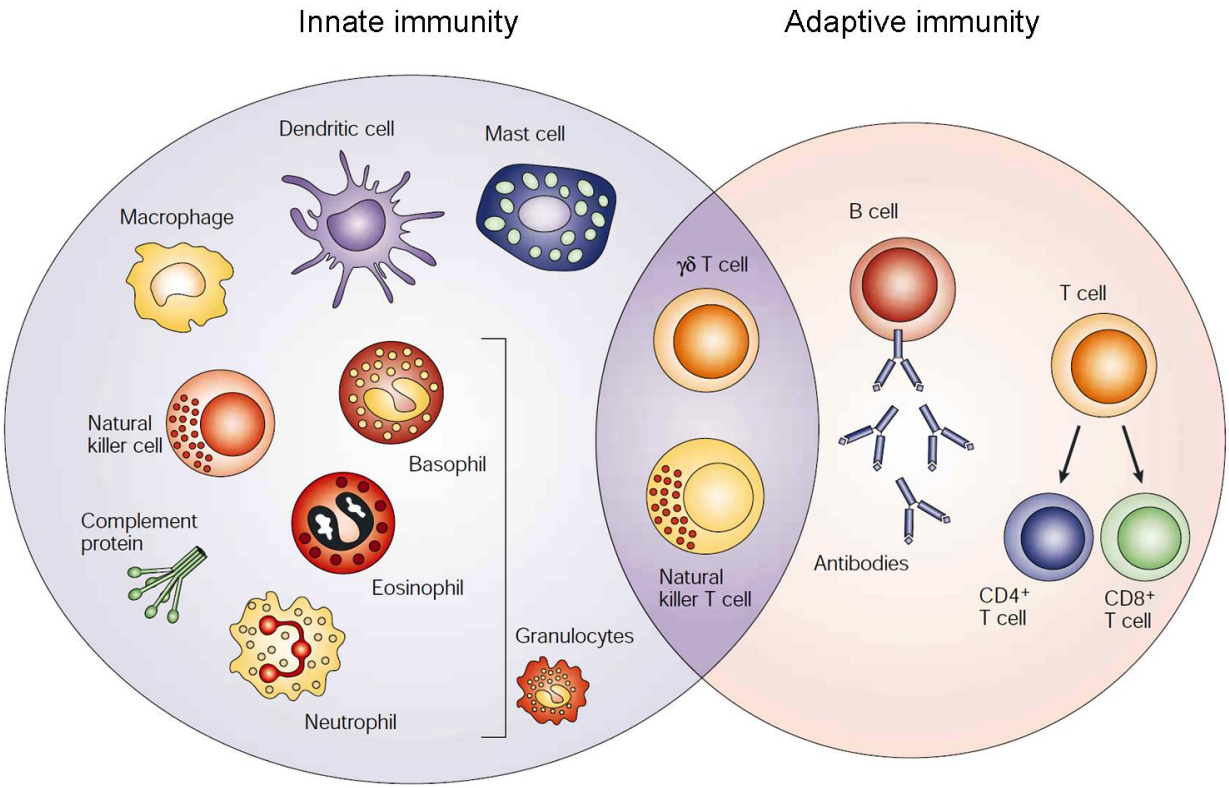

Fig. 1: Cellular components of the immune system. Immune cells are classified as innate or adaptive immune cells based on their function. γδ T cells and natural killer T (NKT) cells are part of both arms of the immune system since they show characteristics of innate immune cells but express T cell receptors which are specific for adaptive immune cells (modified from [3]).

2.1.1 The innate immune system

The task of the innate immune system is the defense against invading pathogens without prior antigen stimulation. This part of the immune system plays an fundamental role in early stages of immune reactions and it is the first line of defense against infection. In fact, without the innate immune system the host would die a few hours after infection. Once a pathogen has crossed the physical barriers, different effector mechanisms are activated. The innate response includes soluble proteins and bioactive small molecules that either directly act on the pathogens (*e.g.* complement proteins, defensins and ficolins) or are released from cells upon activation (*e.g*. cytokines and chemokines), to subsequently recruit and activate additional immune cells [1, 4]. Constituent parts of the innate immune system are hematopoietic cells like macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, natural killer (NK) cells, and natural killer T (NKT) cells [2]. These cells express the so-called pattern recognition receptors (PRR), which are able to recognize highly conserved components of pathogens, the pathogen-associated molecular patterns (PAMPs) [5, 6]. PRR are a large group of receptor molecules, including toll like receptors (TLRs), complement receptors, C-type lectins, and nucleotide-binding oligomerisation domain (NOD) receptors [7-9] and detect a wide range of non-mammalian structural motifs found in and on microbial pathogens (*e.g.* lipopolysaccharides [LPS], dsRNA and certain DNA motifs). The innate immune system can distinguish with these receptors between self and non-self and/or dangerous molecules. Upon sensing potential harmful motifs signaling cascades are started, which result in the activation of an appropriate immune response [1].

Most cells of the innate immune system have the ability to take up pathogenic microbes by phagocytosis or micropinocytosis. Inside the cell the phagosomes are fused with lysosomes to kill the engulfed microbes. Macrophages and especially DCs are able to process the uptaken antigens (Ags) and load the resulting peptides on either major histocompatibility complex class II (MHC class II) or, in case of cross-presentation MHC class I molecules. These MHC molecules are transported then to the cell surface and present the loaded peptides to other immune cells. Due to this function these cells are called antigen-presenting cells (APCs) [10].
DCs are derived from bone marrow progenitor cells and represent a heterogenic group of innate immune cells with subsets, which possess different morphologies,

phenotypes and functions [11]. Immature DCs start a maturation and activation process after being stimulated by either PRRs binding to PAMPs or Ag uptake. During this process the expression of Ag presenting molecules (MHC class I and class II), co-stimulatory molecules (*e.g.* CD40, CD80, CD86, OX40L) and adhesion molecules (*e.g.* CD11a, CD11b, CD54, CD58) is up-regulated [12, 13]. Mature activated DCs induce both innate and adaptive immune responses by the production of cytokines (*e.g.* IL-12, IL-15, IL-18, type I interferons [IFNs], tumor necrosis factor alpha [TNFα]), and the activation and expansion of lymphocytes [14-16]. The ability of DCs to activate a large variety of different immune cells is the key for their in linking the innate and the adaptive immune system.

2.1.2 The adaptive immune system

The innate immune system reacts fast and quite unspecific to everything that fulfills at least one of the requirements “foreign” and/or “dangerous” (see 2.1.1). This response is not only unspecific, but also short lasting in nature. In contrast, the adaptive immune system requires some time to answer to a potential pathogen in a highly specific manner, including the establishment of immunological memory.
The adaptive immune system can be subdivided into two parts, the humoral and the cellular arm, which collaborate to protect the host against threats from inside and outside the body.

The humoral immunity is the defense mechanism of choice against extracellular pathogens and toxins, but is also needed for clearance of certain intracellular pathogens and viruses. Humoral immunity is mediated by immunoglobulins (Ig), also called antibodies (Abs). They are secreted by B cell derived plasma cells upon stimulation/activation. Abs from plasma cells confer protection by (i) neutralization, (ii) opsonization and (iii) complement activation. Neutralizing Abs bind to bacteria and viruses in the extracellular space and prevent their attachment to the cell surface. Bacterial toxins are also fought by neutralizing Abs, which block their biological activity and hinder them to enter host cells [1, 17]. To enhance the killing of pathogens, their surface is coated with Abs to mark them as a foreign target. This process is called opsonisation. The constant region of an Ab binds to the Fc receptor on phagocytic and NK cells which leads either to phagocytosis or in the case of NK

cells to so-called antibody-dependent cell-mediated cytotoxicity. The underlying mechanism is comparable to the action of cytotoxic T lymphocytes (CTLs), involving the release of cytoplasmic granules containing perforin and granzymes [1, 18]. The third mechanism of action of Abs is the activation of the complement system after binding to a pathogen. Complement proteins can enhance the opsonization process, recruit phagocytic cells to the site of infection or even directly lyse the pathogen [1, 7].

Naïve B cells circulate through blood, lymph nodes (LNs) and spleen and express IgM and IgD on their cell-surface, which in turn act as their Ag receptors. When encountering multivalent Ags, the initiation of B cell activation takes place by clustering the Ag receptors. The next step is an increased expression of specific markers (*e.g.* MHC class II, CD80/CD86), which are needed for the subsequent interaction with T helper (Th) cells to deliver the secondary signal for activation. Th cell secreted cytokines are able to stimulate the proliferation of B cells, their differentiation into plasma cells and subsequently the formation of memory cells [1, 19]. After first contact with a pathogen, plasma cells produce IgM isotype Abs, which have normally quite low affinity to the Ag. However, due to the formation of pentamers, IgMs can compensate their low affinity with a high avidity and the ability to trigger efficiently the complement system. B cells can undergo somatic hypermutation, gene conversion and class switch, which subsequently leads to the production of highly specific Abs of the IgG and IgA subclasses [1, 20].

The second part of the adaptive immune system is the T cell mediated immunity. This system consist mainly of immune cells characterized by their α/β T cell receptor (TCR) expression, which recognize peptide Ags bound to MHC class I or II molecules. These α/β-T cells differentiate into different subsets: (i) $CD8^+$ T cells which act primarily via direct killing of cells infected with intracellular microbes, and (ii) $CD4^+$ T cells which orchestrate and regulate the cellular and humoral immune responses. Another small subset of α/β-T cells is characterized by by $CD4^-$ $CD8^-$ $NK1.1^+$ phenotype. The so-called NKT cells recognize glycolipid antigens presented by the CD1d molecule and show immune-regulatory properties by the secretion of cytokines, such as IFNγ, IL-4, granulocyte-macrophage colony stimulating factor (GM-CSF) or TNFs. During the development of T cells the TCR complex is formed by

combination of variable α and β chains. Moreover, each chain can even undergo gene rearrangement which leads to the generation of a huge diversity of different TCRs with specific affinity to almost all possible Ag structures. However, all T cell receptors on one T cell are identical [1, 2].

Naïve T cells need three signals to become activated. First the binding of the TCR and the CD4/8 molecule to the specific Ag presented either on MHC class II for $CD4^+$ or on MHC class I for $CD8^+$ cells. TCRs are also associated with the CD3 complex which transduces the activation signal when the TCR binds to antigen-MHC complexes. Second, T cells require for full activation the binding of co-stimulatory molecules, such as CD28, and as third signal the binding of cytokines released by APC. The combination of all three signals results in the activation of genes that control lymphocyte proliferation and differentiation. All signals for activation of $CD4^+$ T cells can be delivered by APCs like DCs, whereas for the activation of $CD8^+$ T cells an additional support of Th1 cells in most cases is needed. When DCs contact T cells, the so-called immunological synapse (IS) is formed. This is a specialized interface for information exchange where interacting molecules cluster. The DC/ T cell contact normally takes place in highly organized lymphatic tissues like the lymph nodes (LN), where the chances of encounter between T cells with corresponding TCR to Ag presented by DCs is dramatically increased [1, 2, 21, 22]. Activated antigen-specific $CD8^+$ T cells differentiate into CTLs, able to kill infected host cells or cancer cells in a direct contact dependent manner. Thereby, target cells are specifically recognized by CTLs due to the display of Ags on a MHC class I molecule. Upon binding to a target cell, CTLs mobilize cytolytic granules containing mainly perforin and granzymes. The content of the cytolytic granules is secreted and granzymes enter the target cells via perforin-formed pores. Inside the target cell granzymes trigger signal cascades which induce apoptosis in the target cell. In addition, activated CD8+ T cells secrete a variety of cytokines (*e.g.* IFNγ and TNFα) which influence and support the function of other involved immune cells [1, 2, 23, 24].

The largest T cell population is characterized by the $CD4^+$ phenotype. These cells direct and regulate the immune responses after binding to Ag peptides presented on MHC class II molecules on APCs. In Fig. 2 the four main subsets are presented, which can develop from a naïve $CD4^+$ T cell upon encounter of an Ag presented by

APC. The circumstances and mechanisms leading to an antigen specific response are still not completely known and considerable scientific effort is invested into this field. This work focuses on the development of Th subsets, especially the Th17 subset, mainly with respect to the influence of the Ag priming dose. Th1 and Th2 cells are discussed in section 2.1.3, whereas section 2.1.4 focusses on Th17 cells.

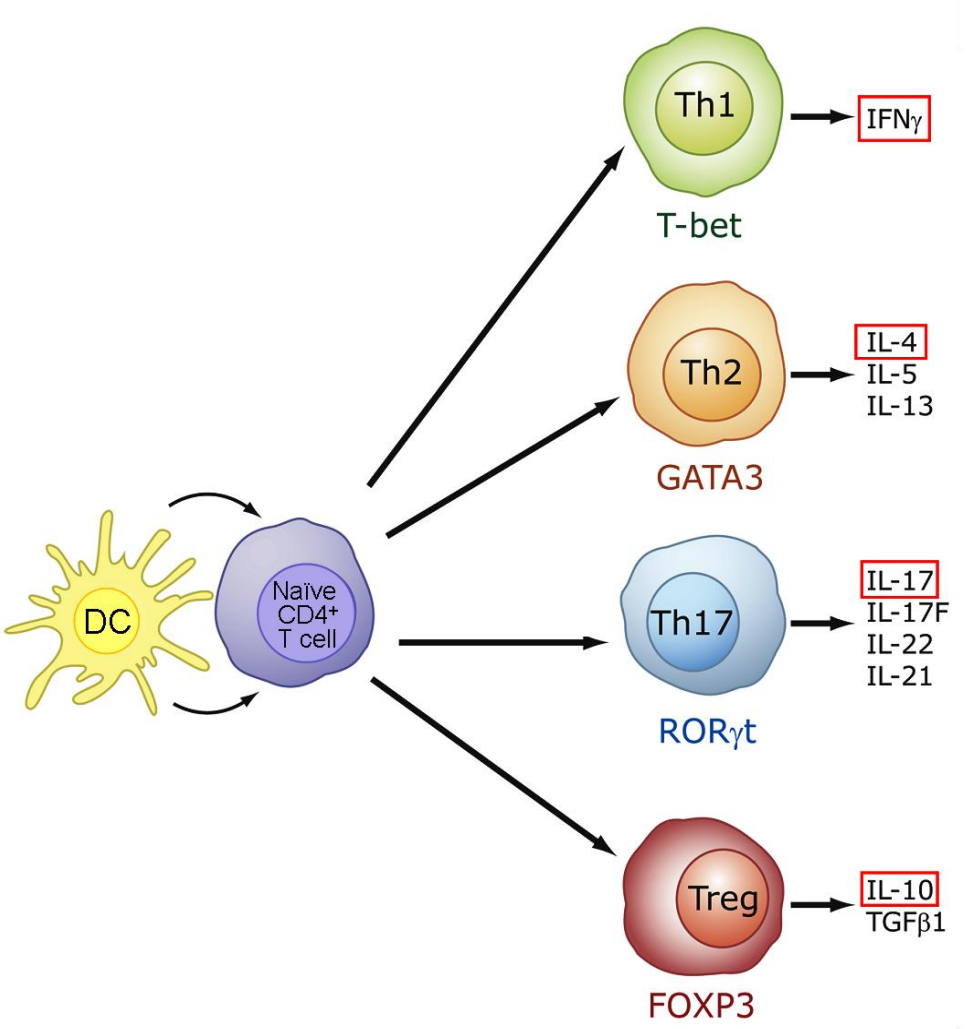

Fig. 2: Differentiation of naïve CD4$^+$ T cells to effector subsets. The possible differentiation routes of a naïve CD4$^+$ T cell after stimulation by Ag loaded DC is shown. Effector subsets are displayed with their lineage specific transcription factor and secreted cytokines. The main cytokines from each subset are marked with a red box (modified from [25].

The regulatory T (Treg) cell subset is characterized by the expression of the transcription factor forkhead box P3 (FOXP3), high expression of CD25 in most cases and the production of IL-10 and TGFβ [26-29]. Their main function is the regulation or modulation of an induced immune response. This results in tolerance and prevents an overreaction of other immune cells, which would lead to severe

inflammations or autoimmune diseases, as observed in individuals with mutations in the FOXP3 gene [28, 30-33]. Furthermore, the establishment of a healthy immune homeostasis in newborns as well as the return to this homeostasis state after clearance of a pathogen is facilitated by Tregs (reviewed in [34]). The interaction of Tregs with Th cells and their regulatory function is discussed in the sections 2.1.3 and 2.1.4.

The differentiation of a naïve $CD4^+$ T cell to a Treg can take place in either (i) the thymus in the presence of self Ag, leading to thymic (t)Tregs or (ii) the periphery in contact with non-self Ags like allergens, food or the commensal microbiota, leading to induced (i)Tregs. The induction of tTregs depends mainly on the strength of TCR signaling and the presence of TGFβ and especially IL-2 in the microenvironment (Fig. 3). Naïve T cells with TCRs which show a high affinity to Ags presented on DCs in combination with low co-stimulation at the same time (*e.g.* potential allergens or Ags from commensal microbes) develop to iTregs (reviewed in [35]).

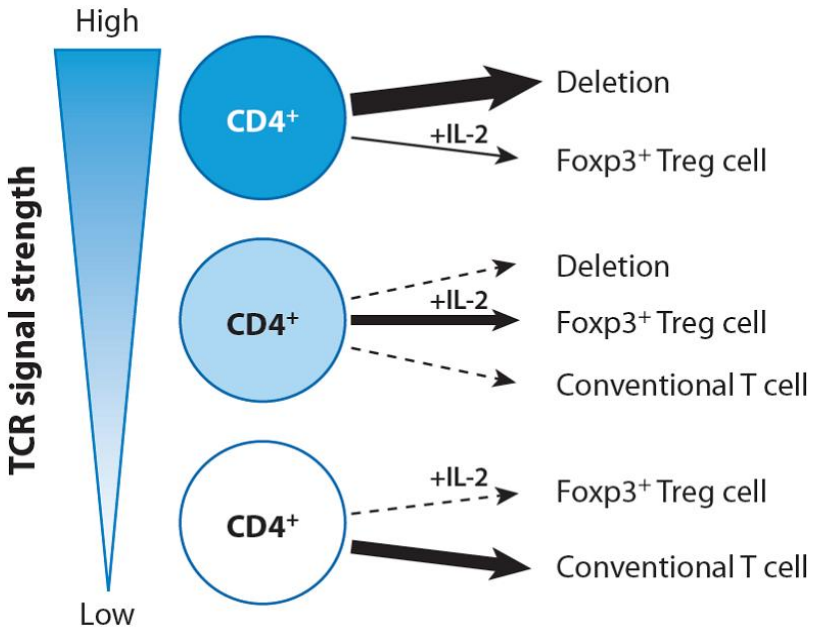

Fig. 3: Treg differentiation in the thymus as a result of TCR signal strength and the availability of IL-2. High TCR affinity to self Ag leads either to depletion of the T cell or differentiation to a Treg. Medium TCR affinity can lead to depletion, often Treg differentiation or a conventional T cell. Low TCR signaling strength elicited by self Ag leads in most cases to conventional T cells and rarely to Tregs. The fate of the T cell is in all three cases controlled by the availability of IL-2. [35]

2.1.3 T helper 1 and T helper 2 cells

T helper cells were first described in 1986 and divided into Th1 and Th2 cell based on their ability to produce either IFNγ or IL-4 [36]. These two cell subsets can be further characterized by lineage specific transcription factors and signal transducers. Th1 cells express T-bet and are dependent on STAT1 and 4 signaling, which are the major transducers in IFNγ and IL-12 signaling [37-42]. Th2 cells express the GATA3 transcription factor and need the support of STAT5 and 6 signaling (IL-2 and IL-4 pathways) for differentiation and effector function [43-47].

As mentioned before, the immune response against intracellular pathogens is mainly controlled and mediated by Th1 cells and their ability to produce IFNγ, IL-2 and TNFα. IFNγ stimulates macrophages to ingest and kill microbes and further promotes production of IgG2a and IgG3 subclasses by B cells in mice. In combination with IL-2, IFNγ activates CTLs and plays a critical role in T cell memory formation, whereas TNFα is a primary inducer of inflammation [48-51]. In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and coordinate the humoral immune response, especially against extracellular pathogens including helminthes. IL-4 mediates the IgE class switch in B cells and is involved in a positive feedback loop in Th2 differentiation, whereas recruitment and activation of eosinophils is controlled by IL-5. The production of IL-10 has anti-inflammatory effects, blocks Th1 differentiation and activates B cells. IL-13 plays an important role in host protection against helminthes and B cell activation. Direct cell-cell contact of Th2 and B cells, together with the secretion of IL-4, IL-10 and IL-13 lead to the activation, proliferation and differentiation of B cells, which in turn results in the production of highly specific neutralizing Abs [1, 49, 52-57]. It is known that some cells express Th1 and Th2 cytokines at the same time in various combinations [58]. However, the numbers of these few “mixed” Th cells are negligible, as compared to the vast majority of clearly discriminable Th cells.

Another point to mention is the fact that abnormal or uncontrolled Th responses can lead to severe health problems like chronic inflammation due to delayed type hypersensitivity caused by Th1 or asthma in the case of Th2 [59, 60]. Thus, the “right” type of Th response is crucial for the host when challenged with a pathogen, as shown by infections with persisting intracellular parasites of the genus *Leishmania.* These parasites can cause leishmaniasis, an illness with symptoms

ranging from small and local cutaneous inflammation, through widespread skin lesions which resemble leprosy, up to systemic infections associated with failure of inner organs and death [61]. Upon infection with *Leishmania major* most inbred mouse strains like C57BL/6 show a Th1 dominated immune response and are resistant or recover very fast from the parasite. In contrast, BALB/c mice fail to establish a protective Th1 immune response. The induced Th2 driven humoral immune response is not protective and leads finally to death of mice [62]. This remarkable example of mounting different Th responses upon infection with the same pathogen illustrates an important challenge in vaccine design. It is not enough to use just the “right” formulation of Ag epitopes, but also to promote the right type of response. Often Ags need to be co-administered with immune modulators or adjuvants to promote an immune response of the appropriate strength and type in order to confer protection. This leads directly to the question of how Th differentiation is induced and controlled.

As described in section 2.1.2, the combination of three signals provided by APCs is required to start activation and differentiation of naïve $CD4^+$ T cells. The key information to understand this process is that all three signals are not just simple events, but rather complex integrated processes in terms of signal strength, duration, composition and interaction [63-65]. The consequence of this is that differentiation of Th cells is mainly controlled by innate immune cells, especially DCs, since T cells only differ in their TCR and their affinity to presented Ags. DCs express many different PRRs which trigger different signaling pathways when binding to PAMPs. Thus, they react with different patterns of co-stimulatory molecule expression and cytokine release in response to different pathogen classes [1, 52, 65, 66]. The influence of cytokines on polarization of Th differentiation is almost elucidated. Th1 cell induction is dependent on the presence of IFNγ and IL-12, and for clonal expansion on IL-2. Once induced, Th1 cells produce IFNγ and IL-2 by themselves which act in an autocrine fashion. In contrast, polarization to Th2 cells mainly requires IL-4 and to a lesser extent IL-2, showing the cells an autocrine IL-4 feedback loop [67-69]. The cytokines secreted by one Th type suppress directly the differentiation of naïve T cells to the other Th phenotypes (*e.g.* IFNγ blocks Th2 differentiation, whereas IL-4 and IL-10 block the induction of Th1 cells) [52, 70]. The cross-regulation of Th1/2 and Th17 cells by cytokines is discussed in section 2.1.4. It

is known that also Tregs are able to influence the induction of Th cells via cytokines. This can be performed by different mechanisms: (i) indirectly, by interacting with DCs to inhibit their production of Th polarizing cytokines [71-73], (ii) by expression of high levels of CD25 (a subunit of IL-2 receptor) to deplete the available IL-2 in the microenvironment [74], and (iii) by release of cytokines (*e.g.* TGFβ and IL-10), which directly block Th differentiation [75, 76].

Beside cytokines, co-stimulatory molecules are responsible for shaping the Th immune response. The major co-stimulatory molecule on T cells for activation, CD28 with its binding partners CD80 and CD86 expressed on APC, was shown to influence Th polarization. Not only the signal strength and duration are important (stromg and long-lasting co-stimulation favors Th2 polarization [77]), but also the ratio between CD80 and CD86 on the APC is important (CD80 directs towards Th1 while CD86 toward Th2 [78]).

Already the strength of the primary signal for T cell activation, binding of TCR to Ag/MHC class II complex itself, directs the fate of a naïve T cell. When talking about signal strength against the background of TCR two points have to be considered: (i) affinity of the TCR to the complex of Ag peptide presented on MHC class II molecule, and (ii) the Ag dose. Naïve $CD4^+$ T cells expressing a transgenic TCR show a Th1 dominated polarization upon stimulation with the corresponding peptide, whereas stimulation with a low-affinity altered peptide ligand (APL) results in a Th2 differentiation [79, 80]. Corresponding results were obtained when changing single residues of the TCR responsible for peptide binding [81]. As expected, also the Ag dose influences the signal strength and, subsequently, also Th development. In 1995, two independent *in vitro* studies with TCR transgenic T cells showed induction of Th2 or Th1 cells when low or high doses of Ag were used, respectively (Fig. 4 and Fig. 5). Hosken *et al.* showed in their study with cells from DO11.10 TCR transgenic mice and OVA-peptide AA323-339 an additional raise of Th2 cells when adding very high Ag doses to the culture (Fig. 5) [82, 83]. An explanation for these findings is the increased Erk activation after strong TCR stimulation. This leads in turn to inhibition of GATA3, consequently weaker Th2 induction, and higher frequencies of Th1 cells [77]. In contrast, the occurrence of Th2 cells at very high Ag doses can be explained by STAT6 independent Th2 differentiation which is driven by high levels of IL-2 and STAT5 signaling [84]. Consistent with these results, *in vivo* studies with immunogenic

peptides demonstrated a Th1 polarization with high and Th2 polarization with low Ag doses [79, 85].

It has to be kept in mind, that the Ag dose will not only affect the amount of presented Ag by DCs but also influence the level of activation and co-stimulatory molecules expressed by DCs. This is even more influenced when the titrated “Ag” is a living pathogen or whole cell extracts, since the amount of delivered PAMPs will also influence the outcome of the immune response [86].

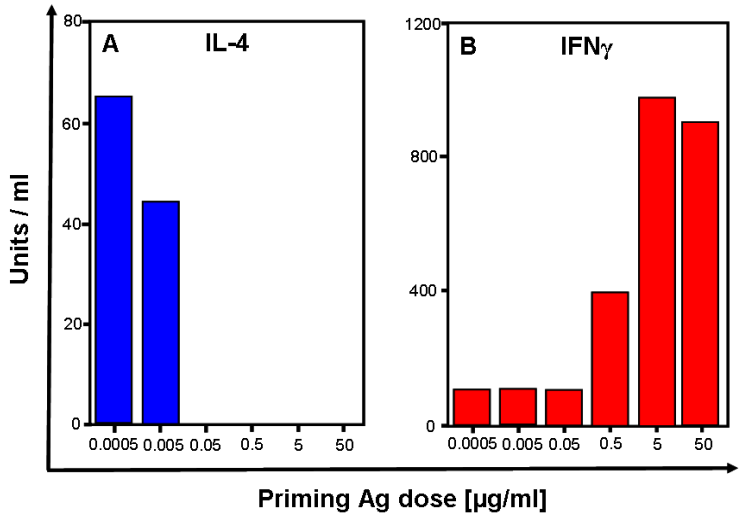

Fig. 4: Cytokine secretion by TCR transgenic CD4$^+$ T cells primed with different Ag doses. Naïve TCR transgenic CD4$^+$ T cells were primed with different doses of the corresponding Ag peptide in the presence of DCs and restimulated after 5 days. ELISA was performed to measure cytokines in the culture supernatant. (A) Amount of secreted IFNγ. (B) Amount of secreted IL-4. (modified from [82])

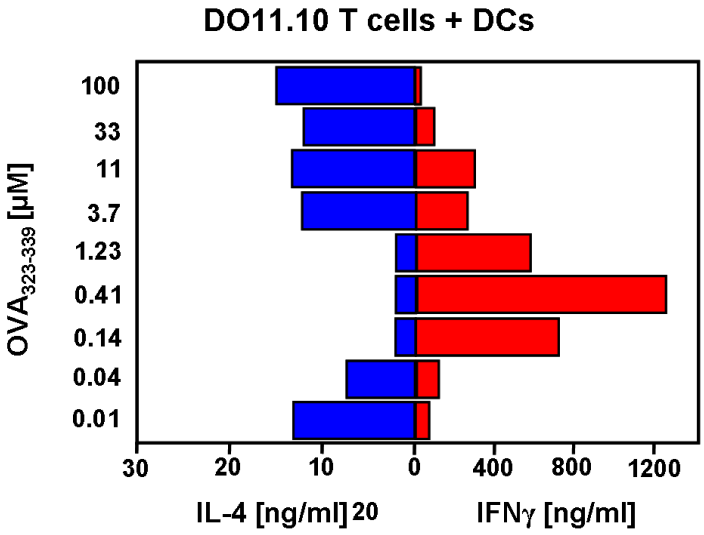

Fig. 5: Cytokines released by TCR transgenic DO11.10 T cells after priming with different dosages of OVA-peptide AA323-339. Levels of IL-4 and IFNγ in cell culture supernatants after re-stimulation of naïve T cells primed with different Ag peptide doses during 5 days in presence of DCs (modified from [83]).

2.1.4 T helper 17 cells

In 2005, the discovery of a 3rd Th subset changed the dogma of regulation of immune responses by Th cells [70]. The new lineage was named Th17 and is characterized by the ability to secrete mainly IL-17A, IL-17F and IL-22 [70, 87]. Thereby, IL-17A and IL-17F are able to form homo- and hetero-dimers (IL-17A/A, IL-17-F/F and IL-17A/F). This study was focused on the action IL-17A/A and IL-17A/F since these members of the IL-17 family cover by far most effector functions and show similar biological properties as IL-17F/F [87, 88]. Thus, “IL-17” will stand for IL-17A in the following, since IL-17A/A and IL-17A/F are recognized by the same Ab against IL17-A.

As the other Th subsets, also Th17 cells show the expression of a lineage characteristic transcription factor, namely RORγt [89] and are dependent on the STAT3 signaling pathway. STAT3 is the major signal transducer for IL-6, IL-21 and IL-23, as well as responsible for the induction of IL-23R and subsequent production of IL-17 [90-92]. The cytokines secreted by Th17 cells fulfill different functions in the host. IL-17 induces many pro-inflammatory molecules, such as IL-6 and IL-8, and has an important role in the induction of inflammatory immune responses [93]. Recruitment of neutrophils as well as the induction of anti-microbial peptides (*e.g.* β-defensins and lipocalin2) is also facilitated by IL-17. Receptors for IL-17 are expressed ubiquitously, with particularly high levels in hematopoietic tissues and in cells of the liver, kidney, prostate and joints [93-95]. IL-22 has bipolar functions; it induces tissue inflammatory reactions, as well as enhanced expression of tissue protective genes and anti-microbial peptides [96]. Experimental data suggest that inflammatory functions are induced in combination with IL-23 [97], whereas protective effects are observed mainly in acute liver and intestine inflammation [98, 99].

Different studies showed that Th17 cells are involved in the induction and progression of many diseases linked to autoimmunity or deregulated immunity (*e.g.* rheumatoid arthritis [RA], multiple sclerosis [MS], inflammatory bowel disease [IBD], asthma and psoriasis) [100]. For example, in RA, IL-17 stimulates by itself and in conjunction with TNFα and IL-1β various cell types to induce inflammation by enhanced production of cytokines, chemokines and destructive mediators, as well as promotion of influx of neutrophils and monocytes, which further increase

inflammatory responses [101-103]. The observed chronic inflammation of joints and bone destruction in RA induced by Th17 cells [101] is in accordance with the increased expression of IL-17R in joints. For MS and the murine homolog experimental autoimmune encephalomyelitis (EAE), it was shown that especially high levels of IL-17A are involved in neuro-inflammation and high clinical scores for disease progression, whereas neutralization leads to delayed disease progression or even partial recovery [104-108]. Although the exact role of Th17 cells in MS/EAE is not yet fully understood, one possible mechanism is the disruption of blood-brain-barrier and subsequently inflammation of the central nervous system as shown by Kebir *et al*. [109]. In IBD the situation is not as clear as in the examples before. Patients with ulcerative colitis or Crohn's disease (two forms of IBD) show high levels of IL17A in the mucosa and serum, corresponding to inflammatory progression [110, 111]. In contrast, in mouse models for IBD the results are contradictory, some studies showed a positive effect of IL-17 neutralization [108, 112], whereas others showed a more severe progression of disease after neutralization of IL-17 or when *Il-17a* knockout mice were used [108, 113]. An explanation for this might be differences in the mouse gut microflora between different labs, which are in turn able to influence the IL-17 pathway during the IBD process [114].

In contrast, Th17 immune responses can also be beneficial or even needed. Protection against many pathogens (*e.g. Candida albicans*, *Borrelia* ssp., *Helicobacter pylori, Klebsiella pneumoniae, Citrobacter rodentium, Salmonella* spp*., Mycobacteria, Bordetella* ssp., *Influenza [115]*) is facilitated or rely on a Th17 directed immune response. Huang *et al.* showed in IL-17RA deficient mice an increased susceptibility to systemic *C. albicans* infection [116]. Patients with hyper IgE syndrome or chronic muco-cutaneous candidiasis have impaired *Candida* specific Th17 responses and showed dramatically increased susceptibility for mucosal *C. albicans* infections [117, 118]. In *Borrelia* infection, Th17 cells are induced and their signature cytokines levels are increased. Th17 cells seem to have a beneficial effect in early protection, whereas IL-17 was shown to lead to more severe destructive arthritis if a chronic *Borrelia* infection was established [119-121]. A clearly protective effect of Th17 cells in mouse model was observed in *K. pneumoniae* infections, where both cytokines, IL-17 and IL-22, were shown to be essential for survival upon challenge [122-124]. A recent study showed the protective effect of transferred Th17

cells upon challenge with an otherwise lethal dose of influenza virus. In addition, the inhibitory influence of IL-10 expression on Th17 development during influenza infection results in a negative effect on survival [125]. Thus, IL-10 as a cytokine produced by Th2 and Tregs is not only able to inhibit the induction of Th1, but also of Th17 cells.

It is known, that the induction of Th17 cells is influenced by different factors, as it is the case for Th1/2 cells (see section 2.1.3). Also for Th17 development, the cytokine milieu has a huge impact. The presence of IL-6 contributes to polarization to Th17 cells and since the IL-6R is expressed at high levels especially in naïve T cells, IL-6 seems to have a key role in Th17 development [65, 126, 127]. In addition the TGFβR is highly expressed in naïve cells and the presence of TGFβ in low levels was shown to polarize naïve cells towards Th17, whereas high levels of TGFβ direct to the Treg lineage [35, 128]. As the other Th subsets, also Th17 cells seem to be regulated by an autocrine feedback loop, which is driven by IL-21 in Th17 cells. This cytokine is not only produced by Th17 cells, but also by many other cell types and is able to promote Th17 development in an autologous way while it blocks the production of IFNγ and hence Th1 polarization [65, 126, 129, 130]. The cytokine IL-23 is not needed for induction but crucial for survival of Th17 cells [131].The differentiation to Th17 cells can be inhibited by all other $CD4^+$ T cell subsets by the production of their main cytokines (IL-2, IL-4, IL-10 and IFNγ), which trigger the signaling pathways that would normally induce Th1/2 or Tregs and subsequently block Th17 differentiation [91, 125, 132-134]. These findings support the fact that uncontrolled Th17 immune responses can lead to severe autoimmunity and chronic inflammation.

Not only cytokines but also other soluble molecules influence Th development. The *all*-trans retinoic acid (ATRA) reduces the expression of IL-6R and IL-23R and is able to induce Tregs development [135-138]. Both effects constrain Th17 cells, but since the receptor for ATRA is not expressed on naïve T cells ATRA does not seem to play a critical role in the initialization of polarization of Th cells, but rather in ongoing Th differentiation [138, 139]. The varying levels of aryl hydrocarbon receptor (AhR) agonists in cell culture medium from different suppliers have strong influence on Th17 polarization, since high levels of Ahr agonists are needed for optimal induction [140]. Beside natural sources, AhR agonists are also found in cigarette smoke and

industrial contaminants and may play a role in the raise of autoimmune diseases, which correlates with the industrial use of hydrocarbons [65, 140].

Beside the cytokines and other soluble factors present in the microenvironment, the binding of co-stimulatory molecules at the time point of T cell activation is important for the fate of naïve T cells. CD28, the main transducer for secondary signaling in T cell activation, is initially needed for Th17 polarization, whereas prolonged CD28 signaling results in inhibitory effects [107, 141]. The binding of the inducible co-stimulatory molecule (ICOS) was shown to induce strong Th17 activation. Interestingly, ICOSL is expressed in high levels on cells of mucosal tissues and it is known that mucosal application of antigen leads to Th17 induction [142]. It is discussed that ICOS-ICOSL interaction is involved in this differentiation process [107, 142-144]. Furthermore, the binding of CD40-CD40L leads to a crosstalk between T cell and DC, and subsequently stimulation of IL-6 production by DCs, an important factor for Th17 induction, as mentioned before [145]. In contrast, earlier studies showed the importance of CD40 co-stimulation in Th1 differentiation [146, 147]. An explanation for these contradictory observations might be the fact that the level of CD40L expression is regulated by the Ag dose *in vitro* [148].

This fact leads to the role of TCR signaling strength in Th17 induction. While the influence of TCR affinity to Ags presented on MHC class II is rather understood for Th1 and 2 cells (see section 2.1.3), no experimental data about Th17 cells is available in this context. The second factor influencing the signal strength is the antigen dose. In 2009, two studies reported that Th17 cells need relatively strong antigenic stimulation for their induction [149, 150]. These findings are in direct contrast to the fact that strong Th17 immune responses are elicited by intranasal application of Ag [142], which is normally linked to a poor uptake of Ag and leads to a reduction of the active Ag dose. Until now, there is no explanation for this dilemma. A recent publication demonstrates that low-strength T cell activation of human cells induce Th17 differentiation [151]. However, these findings were based only on *in vitro* data where cells were either stimulated with αCD3 Abs or with superantigen. Both methods differ from the natural process of TCR binding to peptide Ags presented by MHC class II molecules and can lead to abnormal T cell behavior, due to the triggering of unusual signaling pathways [152].

Taken all records together, the current knowledge on Ag dose dependent induction of Th17 cells, especially in combination with polarizing cytokines, is very limited and needs further investigation. Thus, the aim of this thesis is to answer some of the pending questions:

- Why some studies showed induction of Th17 cells at weak TCR stimulation, whereas others showed the need for high Ag doses?
- In which way polarizing cytokines influence the Th17 differentiation in presence of different Ag doses?
- What is the situation *in vivo* and how can these new insights be used to derive new vaccination strategies?

As mentioned above, B and T cells can form Ag specific memory cells which can be reactivated upon reencounter of the same Ag. The immunological memory is an essential part for the efficiency of the adaptive immune system since it shortens the time to mount a highly specific and powerful adaptive immune response dramatically. This fast response allows the elimination of already encountered pathogens before they can spread and cause damage to the host. This mechanism is used in the process of vaccination and will be explained in the next section.

2.2 Vaccination

Worldwide about 13 million people die every year by the direct aftermath of infectious diseases, being the second leading cause of death [153]. Latest results in science show that infections with certain pathogens can also trigger cardiovascular diseases and even cancer. Taking these results under account would raise the number of victims from infections considerably above the previously mentioned number [154-158].

Nowadays vaccination is still the most effective and cost-efficient method to prevent infectious diseases and the resulting deaths of millions of individuals [159]. The process of vaccination uses the ability of the immune system to form memory cells against already encountered pathogens. By administration of pathogen epitopes (Ags) in a non-harmful formulation the immune system is taught to build up a powerful defense against these Ags. This leads to a protection of the host from

disease when the real pathogen is encountered. The first scientific approach to use this mechanism was undertaken by Edward Jenner in the 18th century. He inoculated healthy humans with material from cowpox lesions, a quite harmless skin disease caused by Cowpox virus. After recovery these persons were then resistant to the life threatening infection with smallpox, caused by Variola virus. Jenner named this process “vaccination”, derived from the Latin root “vaccinus”, meaning “from cow” [160-163]. Louis Pasteur worked in the 19th century on developing safer strategies for vaccination. He introduced the concepts of attenuation, modification through passage and renewed virulence. His work helped to replace person-to-person vaccination with more or less defined vaccines which were less likely to transmit other diseases [164].

The early work from Jenner and Pasteur laid the basis for the control of ten major diseases (*e.g.* smallpox, diphtheria, tetanus, pertussis and poliomyelitis) by vaccination. The biggest success in this context was the eradication of smallpox in 1980 and of Rinderpest, a cattle disease, in 2010 [160, 165, 166]. However, after decades of research and big advantages in developing improved and safer vaccination strategies, many live threatening diseases are still not controlled or are even spreading. Although big efforts are undertaken to develop vaccines against diseases like AIDS, HCV infection or malaria, researchers have not been really successful yet. Another challenging aspect today is the appearance of antibiotic resistances, as in the case of *Mycobacterium* spp. and *Staphylococcus* spp., and the fast spreading of easily transmitted respiratory infections able to lead to pandemic outbreaks like SARS (severe acute respiratory syndrome) caused by a *corona* virus or bird/swine flu caused by influenza viruses. These challenges make the research for new and/or improved vaccines essential, since from the present view vaccination is still the most valuable tool to prevent infectious diseases [159, 167].

Today there are different types of vaccines available. One group represents whole-cell preparations, which can be either a live attenuated form of the pathogen or an inactivated/dead microbe. Attenuation is achieved by recombinant DNA technologies for creating strains which lack specific virulence genes or the more classical method of passaging the pathogen multiple times and selecting less virulent mutants. The advantage of live attenuated vaccines, like the ones used against measles or mumps is their ability to elicit strong humoral as well as cell mediated immune responses

through replication in the vaccinee. It has been shown that attenuated vaccines are save and do not cause a disease in healthy persons. In contrast, inactivated/killed vaccines cannot replicate and elicit in general weaker and shorter lived immune responses. This leads often to the requirement of boost vaccinations to establish a long lasting protective immunity. Inactivated/killed whole cell vaccines are generated by heat or chemical inactivation, or irradiation with UV light or gamma rays. An advantage is their safety even in immune compromised persons [168-172].

The second group of vaccines is composed of well-defined components, which can be purified from microbial components or expressed as recombinant Ags. Subunit vaccines, like the ones against diphtheria, tetanus, hepatitis B virus or papilloma virus belong to this group. The attractiveness of this approach is the low number of immunogenic targets, thereby maximizing the immune response against these molecules. To achieve protection it is essential to identify the main components of a pathogen which are able to confer protective immunity, and to include the identified Ags into the vaccine formulation. The use of well-defined components in the formulation also improves the safety profile of the vaccinates. However, the overall safety features of such subunit vaccines represent also their limitation. Many pathogens alter their surface structures by mutation and escape by this mechanism immune responses directed against single targets [173, 174]. This might represent a problem for pathogens prone to mutation, since a limited number of Ags are induced in the formulation.

All types of vaccines can be administered via different routes. The application route by itself directly influences the immune response in terms of Th phenotype and localization (local and/or systemic). This is especially important, since the first steps of protective defense have to take place at the initial entry site of pathogen invasion [142, 175]. Most of the currently available human vaccines are given by the parenteral/systemic route by injection (*e.g.* subcutaneous, intradermal or intramuscular). These three different injection routes lead to the uptake of Ag by different APC types. While in the skin many resident APCs, like the Langerhans cells are found which are able to initiate strong immune responses, under the skin or in the muscles only few resident APCs are found. Here the Ag is taken up in most cases by transient resident APCs or travels through the lymphatic system to the next draining

LN. In general, parenteral immunization mainly evokes systemic immune responses with very poor local protection. Thus, this route is especially suitable for vaccination against systemic infections, like hepatitis viruses [175].

However, almost all pathogens are either restricted to the mucosal membranes or need to breach them in order to spread and cause systemic infections. Thus, the stimulation of a local immune response at the potential infection site is a clear advantage, since it confers protection by early blocking of colonization and infection. In contrast, systemic vaccination only protects against disease (*e.g.* symptoms), being vaccinated individuals still prone to colonization and able to transfer the agent to other susceptible hosts. This local protection can be achieved by mucosal administration, since this immunization route induces not only local but also systemic immune responses. Especially intranasal application is easy and leads to local protection in the oral and nasal cavity, as well as in the genitourinary tract. Thus, this route can be recommending not only for vaccines against airborne pathogens, but also against sexually transmitted diseases [176-180]. An example for successful development of a vaccine administered by intranasal route is the live attenuated trivalent influenza vaccine [181, 182].

The drawback of intranasal vaccination is the poor immunogenicity of antigens delivered by this route. This is due to different factors: (i) Ag clearance (*e.g.* ciliar activity, entrapment in mucus, degradation by proteases), (ii) the existence of a local tolerogenic milieu, (iii) limited antigen delivery through the mucosa, or (iv) limited occurrence of APCs to take up the Ag. Thus, Ags should be delivered either by systems which are able to breach through the mucosal barrier or co-administered with suitable mucosal adjuvants [183].

2.3 Adjuvants

As described in section 2.2, Ags in defined subunit vaccines are less immunogenic when compared with live or whole cell component vaccines, which have built-in adjuvant properties. Beside the protective Ags, whole cell vaccines also encompass components acting as danger signals which activate the immune system [184]. Thus, weak immunogenic subunit vaccines need the help of immune stimulatory molecules,

the so-called adjuvants (lat.: adjuvare = to help) to enhance immunogenicity. Almost all subunit vaccines in the market include these molecules (*e.g.* alum, MF59 and ISCOM). An ideal adjuvant should strengthen, speed up and direct the immune response against the applied Ags in a well-defined manner, without having by itself immunogenic or reactogenic properties. In addition, it should be stable with respect to storage but also biodegradable, cheap and fast to produce [185].

Since adjuvants are a complete heterogeneous group of compounds they differ in the mechanisms by which they act and interact with different immune cells. One way is the creation of an antigenic reservoir which slowly releases the Ags over time (*e.g.* alum). In addition, adjuvants can act directly, for example by targeting Ags to APCs and subsequent promotion of Ag processing and presentation. Alternatively, adjuvants can also work indirectly by promoting a microenvironment which helps to modulate, enhance and direct the immune response against the Ags [174].
In general adjuvants are divided into two classes, immune stimulants and vehicles or delivery systems. Immune stimulants like saponins, TLR ligands or bacterial toxins increase the response to Ags by acting directly on the innate immune system. In contrast, vehicles and delivery systems like mineral salts, emulsions or virosomes provide an optimized presentation of Ags to the immune system [172].

At the moment there are only eight formulations approved for use in human vaccines. These include alum, squalene-based emulsion, glycolipide monophosphoryl lipid A derived from LPS, cholera toxin subunit B, virus like particles and influenza virosomes [172, 174]. Since none of the approved adjuvants is “ideal”, there are some disadvantages like the low efficient induction of mucosal immune responses or the poor induction of CTL-mediated immunity [172, 186]. For these reasons, it is still important to search for new adjuvants which are able to overcome the described problems. In the pipeline of industry and research are at the moment new promising compounds, among others synthetic oligodesoxynucleotides containing unmethylated CpG motifs to trigger TLR9, IC31 that also triggers TLR9, MALP-2 a TLR2/6 ligand or saponins [172, 174, 187-191].

An interesting adjuvant with respect to the elicited Th phenotype in the immune response and its mechanism of action is α-galactosylceramide (αGC). This glycolipid

was first derived from the marine sponge *Agelas mauritianus* and later modified by pegylation to enhance its biological properties [192, 193]. It was shown that αGC has immune modulatory properties, leading to the activation of various cell subsets of the innate and adaptive immune system. APCs can present αGC by CD1d molecules to Vα14+ NKT cells which are activated upon binding and start to produce large amounts of IFNγ and IL-4 [194-198]. It was shown that activation of $V\alpha14^+$ NKT cells leads to a Th2 biased immune response and blocks Th17 polarization [199].

Other interesting candidates are the cyclic-di-nucleotides [200-202]. Among them, the bis(3',5')-cyclic dimeric adenosin mono phosphate (c-di-AMP), which was first described as a second messenger signaling for DNA integrity in *Bacillus subtilis* during sporulation [203]. Chemically seen, it is a small cycle of RNA, containing two bases linked to ribose and phosphate. Since c-di-AMP does normally not occur in higher organisms it acts as a PAMP and is sensed by binding to STING [201, 203, 204]. It was shown that c-di-AMP promotes a local and systemic immune response with a Th1/Th17 dominated immune response when used as adjuvant in a murine system [202]. However, in contrast to CpG motifs, a robust Th2 component is still present.

Influenza virosomes seem to be a promising combination of Ag carrier and immune stimulating adjuvant leading to local and systemic protection [205, 206]. For the production of virosomes, an influenza vaccine strain is propagated in chicken eggs and then inactivated (*e.g.* with beta-propiolactone or formaldehyde). Subsequently the surface glycoproteins and phospholipids are solubilized with the detergent octaethyleneglycol onododecylether and the influenza surface antigens hemagglutinin (HA) and neuraminidase (NA) are purified and mixed with lecithin. By step-wise removal of the detergent virosomes form spontaneously and the HA and NA viral antigens are incorporated into the phospholipid bilayer [207]. Similar to influenza virus, virosomes bind to host cells via HA and trigger endocytosis. Inside the endosome, the low pH value leads to HA-mediated membrane fusion and partial escape of virosomes to cytoplasm. Thereby, MHC class I restricted immune responses are stimulated. In parallel, HA and NA fragments are presented by MHC class II molecules and trigger $CD4^+$ T cell activation [205].

3 Aim of the study

Most vaccines on the market trigger by a combination of Ag(s) and adjuvant in their formulation strong Th2 dominated immune responses to provide protection. As above mentioned, a newly discovered Th subset called Th17 has been reported to play an important role in combating certain infectious pathogens. However, their induction by vaccines is poorly understood and it was therefore not considered in the rational process of vaccine design. In this context, it has been shown that the antigen dose plays an important role for shaping Th1/Th2 subsets following immunization.

Thus, the overall aim of this thesis was to clarify the impact of Ag dose on Th17 induction and to investigate whether changes in Ag concentration of vaccine formulations can be exploited to tailor T cell responses. To this end, *in vitro* experiments with cells from TCR transgenic and wild type mice were performed to characterize Ag dose dependent induction of Th17 cells. The experiments investigated polarization (i) under normal culture conditions, (ii) in the presence of a polarizing cytokine milieu or (iii) by neutralization of Th17 blocking cytokines. *In vivo* immunization studies with the model Ag OVA were also performed to set up a model system of targeted Th17 induction. This system was subsequently transferred to a vaccination relevant setting in which the impact of Ag dose-dependent modulation of Th17 responses on protective immunity was addressed *in vivo.* This was achieved by using an influenza vaccine in combination with a murine viral challenge model. To obtain a complete *in vivo* characterization of the T cell responses, a complex 9-color staining panel for flow cytometry analysis was established during this study.

It has been described that Th17 immune responses can correlate with detrimental outcome for the host after infection with certain pathogens. Therefore, the possibility to selectively shutdown Th17 induction after vaccination could represent a true asset for the development of certain vaccines. The adjuvant αGCPEG is able to induce Th2 immune responses, but blocks Th17 induction. However, the mechanism of action was unknown. Thus, the second part of this work investigated the underlying principles of Th17 blockage by αGCPEG, as alternative approach to dose dependent fine-tuning of the elicited responses. To this end, *in vitro* experiments with murine NKT cells and *in vivo* immunization studies with wild type and NKT KO mice were

performed, which enable to characterize the underlying mechanisms of Th17 blockage. It is expected that the knowledge and strategies established during this work will facilitate the development of vaccines able to stimulate tailored Th17 responses.

4 Material

4.1 Antibodies

Table 1: List of used antibodies

Antigen	Clone	Fluorochrome	Company	Cat.#
CD3	500A2	V500	Becton, Dickinson and Company (BD), USA	560773
CD3	17A2	APC	eBioscience (eBio), Germany	51-0032-82
CD3	145-2C11	FITC	BD, USA	553062
CD3	17A2	unlabeled	eBio, Germany	16-0032-86
CD4	RM4-5	PE-Cy7	eBio, Germany	25-0042-82
CD4	RM4-5	APC-Alexa750	eBio, Germany	27-0042-82
CD8a	53-6.7	PB	eBio, Germany	48-0081-82
CD8a	53-6.7	FITC	BD, USA	553031
CD8a	53-6.7	PE	BD, USA	553033
CD11b	M1/70	PE	BD, USA	557397
CD11c	N418	FITC	eBio, Germany	53-0114-82
CD11c	N418	PE	eBio, Germany	12-0114-83
CD11c	N418	APC	eBio, Germany	17-0114-82
CD11c	N418	APC-Cy7	eBio, Germany	25-0114-82
CD25	PC61	PE	BD, USA	553866
CD28	37.51	unlabeled	eBio, Germany	16-0281-86
CD44	IM7	APC	eBio, Germany	17-0441-83
CD45R (B220)	RA3-6B2	PE	BD, USA	553089
CD49b (DX5)	DX5	PE	BD, USA	553858
CD62L	MEL-14	FITC	BD, USA	553150
CD80	16-10A1	PE	eBio, Germany	553769
CD80	16-10A1	APC	eBio, Germany	17-0801-82
CD86	GL1	FITC	BD, USA	553691

CD86	GL1	PE	BD, USA	553692
CD90.1	HIS51	PE-Cy5	eBio, Germany	15-0900-82
CD90.1	HIS51	PE-Cy7	eBio, Germany	25-0900-82
CD90.1	HIS51	PerCP-Cy5.5	eBio, Germany	45-0900-80
CD90.2	53.2-1	FITC	BD, USA	010041
CD90.2	53.2-1	PE-Cy7	eBio, Germany	25-0902-82
I-$A^{b/d}$	M5/114.15.2	eFluor450	eBio, Germany	48-5321-82
IFNγ	XMG1.2	PE	eBio, Germany	12-7311-82
IFNγ	XMG1.2	Alexa647	eBio, Germany	51-7311-82
IFNγ	XMG1.2	PB	eBio, Germany	48-7311-82
IFNγ	R4-6A2	unlabeled	eBio, Germany	16-7312-85
IL-2	JES6-5H4	APC-Cy7	BD, USA	560547
IL-2	JES6-1A12	unlabeled	eBio, Germany	16-7022-82
IL-4	11B11	APC	eBio, Germany	17-7041-82
IL-4	11B11	unlabeled	eBio, Germany	16-7041-85
IL-10	JES5-16E3	AlexaF700	eBio, Germany	56-7101-82
IL-17A	eBio17B7	FITC	eBio, Germany	11-7177-81
IL-17A	eBio17B7	PE	eBio, Germany	12-7177-81
IL-17A	eBio17B7	Alexa647	eBio, Germany	51-7177-82
IL-17A	TC11-18H10	V450	BD, USA	560522
IL-17F	eBio18F/0	PE	eBio, Germany	12-7471-80
IL-17F	eBio18F/0	Alexa647	eBio, Germany	51-7471-80
IL-22	1H8PWSR	PerCP-eF710	eBio, Germany	46-7221-82
NK1.1	PK136	APC	eBio, Germany	17-5941-82
TNFα	MPG-XT22	eFluor450	eBio, Germany	45-7321-82
TNFα	MPG-XT22	PerCP-eF710	eBio, Germany	46-7321-82
Vβ5.1/5.2	MR9-4	PE	BD, USA	553190

4.2 Chemical compounds and reagents

Table 2: Chemical compounds and reagents

Chemical:	**Company:**
3-amino-9-ethyl-carbazole (AEC substrate kit)	BD Pharmingen, USA
Acetic acid (CH_3COOH)	Merck, Germany
Acetonitrile (CH3CN)	Sigma-Aldrich, Germany
Ampuwa®	Serumwerk, Germany
Avidin-HRP (horseradish peroxidase) conjugated	BD Pharmingen
Ammonium chloride (NH_4Cl)	Merck, Germany
Bovine serum albumin (BSA)	Sigma-Chemie, Germany
Brefeldin A	Sigma-Chemie, Germany
Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)	Invitrogen, Germany
Citrat acide –1- hydrate ($C_6H_8O_7 * H_2O$)	Riedel-de Häen
Concanavalin A from *Canavalia ensiformis*	Sigma-Aldrich, Germany
DMEM medium (low glucose)	Gibco, UK
N,N-Dimethylformamide (DMF)	Sigma-Aldrich, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Fluka, Switzerland
Ethanol 100%	Fluka, Switzerland
Fetal calf serum, South America (FCS)	Greiner Bio-One, USA
Formaldehyde ≥ 36.5%	Riedl-de-Haën, Germany
Gentamycine (50mg / ml in deionised water)	Gibco, UK
L-Glutamine	Gibco, UK
Granulocyte-macrophage colony stimulating factor (GM-CSF)	BD, USA
Ionomycin	Sigma-Aldrich, Germany
Isofluran®Curamed vet inhalation anesthetic	Essex Tierarznei, Germany
Isopropanol	Merck, Germany
Isoton II (acid-free balanced electrolyte solution)	Beckman Coulter, USA
2-Mercaptoethanol (50 mM)	Gibco, UK
[methyl-^{3}H] Thymidine solution	Amersham, Germany
Paraformaldehyde (PFA)	Merck, Germany

Penicillin/Streptomycin (100 units/ml Penicillin G Sodium; 50 µg/ml Streptomycin Sulfate in 85% Saline)	Gibco, UK
Phorbol 12- myristate 13-acetate (PMA)	Sigma-Aldrich, Germany
Potassium chloride (KCl)	Fluka, Switzerland
Potassium hydrogen carbonate ($KHCO_3$)	Merck, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Carl-Roth, Germany
RPMI 1640 medium (+L-glutamine)	Gibco, UK
Sodium acetate (CH_3COONa)	Merck, Germany
Sodium carbonate (Na_2CO_3)	Carl-Roth, Germany
Sodium chloride (NaCl)	Carl-Roth, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Germany
di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
Sodium hydroxide (NaOH)	Carl-Roth, Germany
Streptavidin–horseradish peroxidase conjugate (ELISPOT)	BD Pharmingen, USA
[methyl-^{3}H]Thymidine 5.0 Ci / mmol	Amersham, Bioscience
Trypan blue	Sigma-Aldrich, Germany
Tween 20	Carl-Roth, Germany

4.3 Solutions and buffers

Table 3: Solutions and buffers

Solution/buffer:	**Composition:**
ABTS-solution	0.3 g/l ABTS in 0.1 M citric acid
ABTS / H_2O_2	0.03% (v/v) H2O2 in ABTS-solution
Acetate solution (0.1 M)	148 ml acetic acid 0.2 mM to 352 ml of 0.2 mM sodium acetate; adjust volume to 1 l with water, pH 5.0
ACK lysis buffer	0.1 mM EDTA; 1 mM $KHCO_3$; 155 mM NH_4Cl; pH 7.3
Alsever's solution	2.05% Glucose, 0.8% sodium citrate, 0.42% sodium chloride, 0.055% citric acid/H_2O (w/v)
Blocking buffer (ELISpot)	2% BSA/PBS (w/v)
Dilution buffer (ELISpot)	2% BSA/PBS (w/v)
MACS buffer	0.5% BSA; 2 mM EDTA in PBS
Permeabilization buffer	0.5% BSA; 0.5% Saponin/PBS (w/v)
2% PFA	2% PFA/PBS (w/v), pH 7.0 (fixation)

PBS (phosphate-buffered saline)	2.7 mM KCl; 1.8 mM KH_2PO_4; 137 mM NaCl; 10 mM Na_2HPO_4; pH 7.4
Trypan blue solution	0.1% Trypan blue/PBS (w/v)

4.4 Antigens and adjuvants

Table 4: Antigens and adjuvants

Antigen / Adjuvant:	**Company:**
S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxy polyethylene glycol (BPPcysPEG)	HZI, Germany
Cholera toxin B subunit (CTB)	Sigma-Aldrich, Germany
CpG ODN 1826	MOLBIOL, Germany
Curdlan	Sigma-Aldrich, Germany
Cyclic-di-adenosine-mono.phosphate (c-di-AMP)	HZI, Germany
Cyclic-di-guanosine-mono-phosphate(c-di-GMP)	HZI, Germany
α-galactosylceramide polyethylene glycol (αGCPEG)	HZI, Germany
H5N1 Virosomes	Berna Biotech, Switzerland
HA peptide AA110-120 from influenza virus PR8	HZI, Germany
ISCOM	Berna Biotech, Switzerland
Lipopolysaccharide (LPS)	Sigma-Aldrich Germany
EndoGrade Ovalbumin	Hyglos, Germany
Ovalbumin, grade VII	Sigma-Aldrich, Germany
Ovalbumin peptide AA323-339	HZI, Germany
Ovalbumin peptide AA323-339-FITC	HZI, Germany

For some experiments Ovalbumin-peptide AA323-339 was synthesized with a seven AA “spacer” at the C-terminal end. The C-terminal end of the spacer has been labeled with FITC (Fig. 6).Mutated FITC labeled peptides were generated by exchanging AA335 or AA336, for sequences see Table 5.

Table 5: Sequences of mutated OVA-peptides

Name:	**AA exchange:**	**Final sequence:**
335A	335N →335A	ISQAVHAAHAEIAEAGR
335Q	335N →335Q	ISQAVHAAHAEIQEAGR
336A	336E →335A	ISQAVHAAHAEINAAGR
336D	336E →335D	ISQAVHAAHAEINDAGR

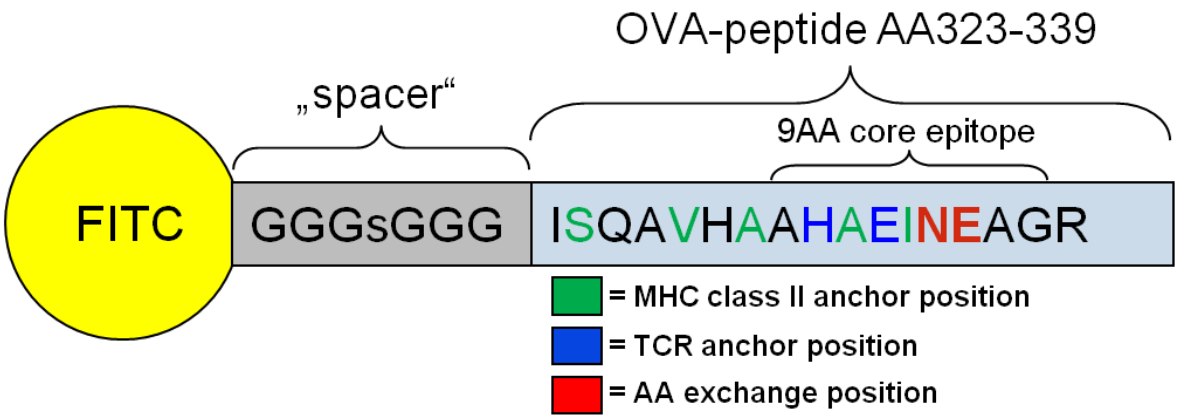

Fig. 6: Schematic structure of FITC labeled OVA-peptide AA323-339. The OVA-peptide (light blue box) is linked by a seven AA long spacer (grey box) to a FITC molecule (yellow circle).

4.5 Consumables

Table 6: Consumables

Product:	**Company:**
15 and 50 ml Cellstar®tubes	Greiner bio –one, Germany
Cell culture plates	TPP, Switzerland
Cell strainer 100 µm	BD Bioscience, USA
CombiTips 1-10ml	Eppendorf, Germany
Cell trics 50 µm filter	Partec GmbH, Germany
Counter cuvettes	Beckman Coulter, Germany
FACS tubes 1.2 ml	MP Biomedicals, France
FACS tubes 5ml	BD Bioscience, USA
Injection needle 1ml 30G x ½“ Omnican F	B. Braun, Germany
Multistepper Matrix	Thermo Scientific, Germany
Parafilm	American National Can, USA
Pasteur Pipettes	Brand GmbH + Co. KG, Germany
Petri dishes	Greiner Bio-one, Germany
Pipette Tips	Greiner Bio-one, Germany
Serological Pipettes 5/10/25 ml	Roth, Germany
Safe lock reaction tubes	Eppendorf, Germany
Scissors and forceps	B Braun, Germany
Tissue culture flask 75/150 cm^3	TPP, Switzerland
VALO SPF eggs	Lohmann Tierzucht GmbH, Germany

4.6 Cell culture media

Table 7: Cell culture media

Medium:	Composition:
RPMI complete	RPMI 1640 supplemented with 10% heat inactivated FCS, 100 U/ml of penicillin, 50 µg/ml of streptomycin and 1 mM L-glutamine
BMDC medium	RPMI complete supplemented with 50 µg/ml gentamycine

4.7 Instruments

Table 8: Instruments

Instrument:	Company:
Centrifuge Biofuge pico	Heraeus, Germany
Centrifuge Megafuge R40	
Centrifuge Multifuge 3S-r	
CTL Immunospot analyzer	CTL-Europe GmbH, Germany
Cell counter Z2	Beckman Coulter, Germany
ELISA Reader Synergy2	BioTek Laboratories GmbH, Germany
Flow Cytometer Aria2, Canto, LSRII	BD, USA
Freezer and Fridge	Liebherr, Germany
Cell harvester ICH-110	INOTECH, Switzerland
Handystep	Brand, Germany
Heating magnetic stirrer	IKA Labortechnik, Germany
Incubator	Heraeus, Germany
Micropipettor	Hirschmann Laborgeräte, Germany
pH-meter	Hannah Instruments, Germany
Pipettbay	Thermo Scientific, Germany
Precision 2000 automated microplate pipetting system	BioTek laboratories, Germany
Plate washer ELx 405	
Scale TE1502S	PerkinElmer, Finland
Scintillation Counter Wallac 1450	
Sterile hood Hera Safe	Thermo Scientific, Germany
Vortex Genie-2	Omnilab, Germany
Water bath	Köttermann, Germany

4.8 H5N1 Influenza virus

The used influenza virus strain NIBRG-14 displays H5N1 and is a vaccine reference strain originally created by the National Institute for Biological Standards and Control (NIBSC) by using reverse genetics to combine A/Vietnam/1194/2004 (H5N1) virus (in which the polybasic HA cleavage site has been excised) and A/PR/8/34 (H1N1) virus. The used technique is described in detail for creation of a similar strain in [208]. The virus used for experiments was propagated and titrated as described in section 5.5.

4.9 Mice

Six to eight weeks old female BALB/c and C57BL/6 mice were purchased from Harlan Winkelmann GmbH (Borchen,Germany). OTII, DO11.10, TCR-HA and C57BL/6 Thy1.1 mice were bred in-house at the HZI animal facility. OTIIxThy1.1 mice were generated by crossing at the HZI.

Jα281 knock out (KO) mice were kindly provided by the animal facilities of the Max Planck Institute for Infection Biology (Berlin).

Mice were treated in accordance with local and European Community guidelines and kept under SPF conditions in individual ventilated cages with food and water *ad libitum*. Experiments using animals were done with the permission of the local authorities; permission numbers: 33.11.42502-04-104/07/2007 and 33.11.42502-04-105/07/2007 from the local government (Bezirksregierung Braunschweig).

Table 9: Phenotypes of used transgenic mouse strains

Name:	Background:	Phenotype:	Reference:
DO11.10	BALB/c	$CD4^+$ T cells express only T cell receptors with affinity for OVA-peptide AA323-339	[209]
OTII	C57BL/6	$CD4^+$ T cells express only T cell receptors with affinity for OVA-peptide AA323-339	[210]
TCR-HA	BALB/c	$CD4^+$ T cells express only T cell receptors with affinity for HA-peptide AA110-120 from influenza strain PR8	[211]
Thy1.1	C57BL/6	All T lymphocytes express CD90.1 in contrast to wild type mice that express CD90.2; *e.g.* congenic marker	[212]
Jα281 KO	BALB/c	By the KO of Jα281 gene, cells of these mice cannot express NKT cell receptor and thus lack functional NKT cells	[213]

5 Methods

5.1 Cell isolation

In all experiments mice were sacrificed by CO_2 inhalation or cervical dislocation.

5.1.1 Spleen and lymph nodes

Spleen and/or lymph nodes (LNs) were removed and minced with the piston of a 3 ml syringe through a 100 µm nylon mesh to obtain a single cell suspension. The erythrocytes were lysed by ACK buffer for 1-2 minutes, followed by a washing step. To remove dead cell clumps, the cell suspension was filtered again though a 100 µm nylon mesh.

5.1.2 Bone marrow

For generating bone marrow derived dendritic cells (BMDCs), hind legs were removed and cleaned from muscles and flesh. The intact bones were disinfected with ethanol for 1-2 min. In a clean bench, both ends of every bone were cut and the bone marrow was flushed out with medium by using a syringe. The bone marrow was disrupted with a syringe to obtain a single cell suspension. After lysis of erythrocytes with ACK buffer the cell number was adjusted to $1x10^6$/ml by diluting the cells in full RPMI supplemented with Gentamycine. To differentiate the cells to DC like cells, GM-CSF (5 ng/ml) was added and cells were cultured in 6 well plates (5 ml/well) for 5-6 days in the incubator. Beginning from day 3, 2 ml of medium per well were exchanged daily. After 5-6 days cells were fully differentiated and were used as DCs.

5.2 Antibody staining for flow cytometry

5.2.1 Surface antigens

The staining of surface antigens was performed in PBS on ice. A pre-diluted mix of all antibodies plus a live/dead staining dye was added to the suspended cells, which were then vortexed. After 30 min at 4°C in the dark, staining was stopped with 4-10 volumes of PBS.

5.2.2 Intracellular cytokine staining

When intracellular cytokines were stained, Brefeldin A was used to block the cytokine transport from the endoplasmic reticulum (ER) to the Golgi apparatus. By this treatment cytokines accumulated in the ER were stained after permeabilization of the cell membrane. In brief, cells were fixated with 2% paraformaldehyde (PFA) in PBS for at least 30 min at 4°C. Then cells were permeabilized by 30 min incubation in permeabilization buffer (perm. buffer). After centrifugation and decanting the supernatant (SN), the Abs mixtures pre-diluted in perm. buffer were added. After 30 min at 4°C in the dark, staining was stopped with 4-10 volumes of perm. buffer. For reducing the unspecific background, an additional washing step with perm. buffer flushed unbound Abs out of the cells. After washing cells one time with PBS they were ready for analysis.

5.2.3 Cell labeling with carboxyfluorescein succinimidyl ester (CFSE)

Cells were labeled with CFSE for quantification of proliferation and discrimination of undivided and divided cells by flow cytometry,. The labeling was performed with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), which can easily pass cell membranes due to its acetate groups. Inside the cell, CFDA-SE is converted by esterases to CFSE and binds covalently to intracellular molecules [214]. While CFDA-SE has no fluorescent properties, CFSE can be excited with a blue laser and measured in the FITC channel.

With every division of a cell, all molecules are equally distributed to the two daughter cells, halving the amount of CFSE molecules in the next generation. This reduction of fluorescent molecules is detected by a flow cytometer as a 50% loss of signal strength. When the CFSE signal of proliferated cells is displayed as a histogram, as shown in Fig. 7A, a typical pattern is seen - every peak from right to left represents one round of division, starting with undivided cells. With the FlowJo software it is possible to calculate the number of cell generations and to mark these in the histogram (Fig. 7B).

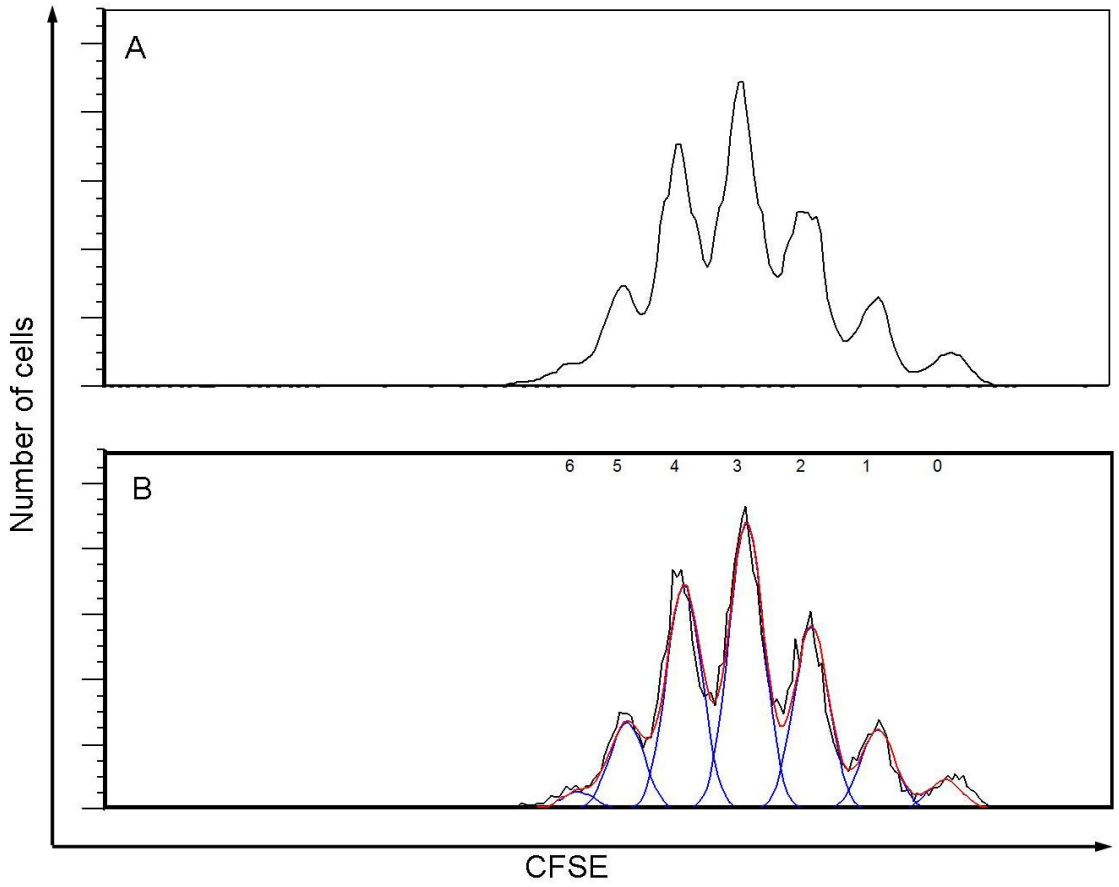

Fig. 7: Histogram showing cell proliferation tracked by CFSE dilution. A: Peak pattern as detected directly by LSR2 **B:** Peak pattern with marked cell generations calculated by FlowJo software

5.3 Flow cytometry

5.3.1 Fluorescence Activated Cell Sorting (FACS)

In most experiments pure and well-characterized cell populations sorted by FACS were used. When fluorochromes with overlapping spectral properties were used in the same staining, spectral overlap compensation was performed by using single stainings for every used antibody.

5.3.1.1 Naïve non-activated $CD4^+$ T cells

For sorting of naïve non-activated $CD4^+$ T cells, single cell suspensions from spleen and LNs were stained for CD4, CD25, CD62L and, in case of C57BL/6 background of the donor mice, also for CD44. The cells were sorted with a BD FACS Aria 2, according to the shown gating strategy (Fig. 8). The first gate (P1) selected total lymphocytes by forward scatter area (FSC-A) and side scatter area (SSC-A). Single

cells were discriminated from doublets with gate two (P2) and three (P3) by FSC-A against FSC-heights (FSC-H) and SSC-A against SSC-width (SSC-W), respectively. The next gating on cells positive for CD4 ($CD4^+$; PE-Cy7) and expressing high levels of CD62L ($CD62L^{hi}$; FITC) selected the naïve CD4 population (P4). Cells negative for CD25 ($CD25^-$; PE) and with low expression of CD44 ($CD44^{lo}$; APC) were non-activated as shown in the last gate (P5). In total the cells were sorted for $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ with purity higher than 99%.

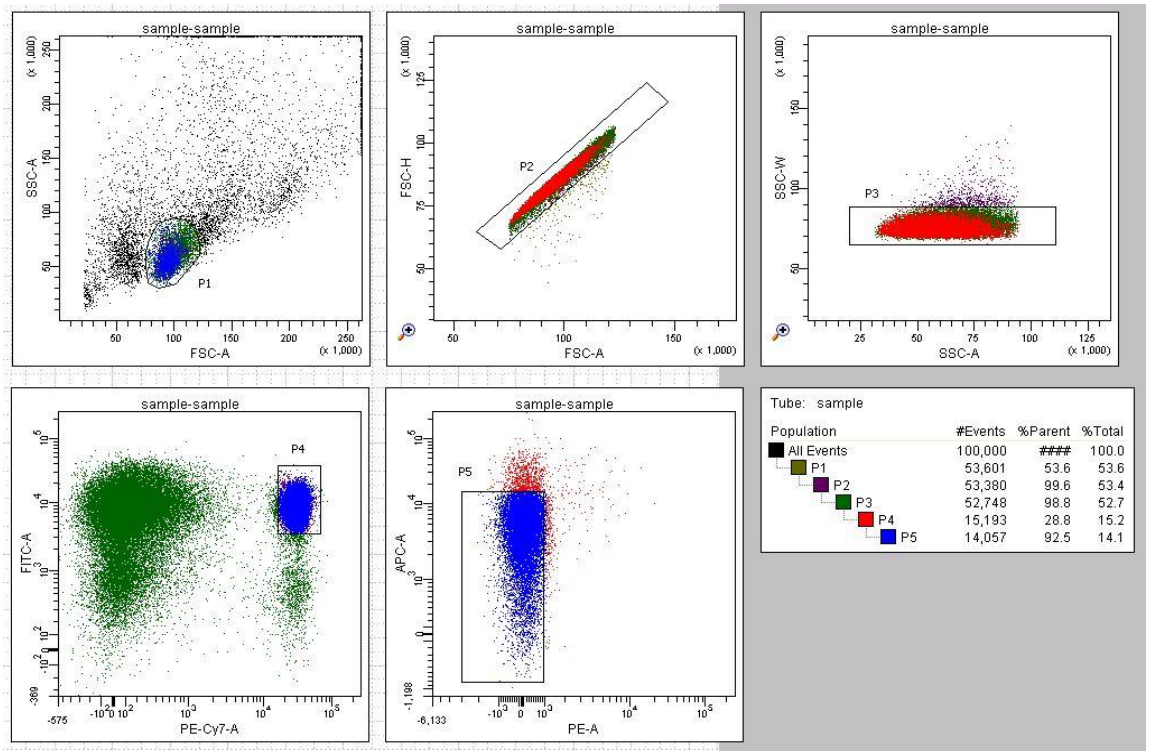

Fig. 8: Gating strategy for sorting of naïve non-activated $CD4^+$ T cells from OTII mice. Screen shots from FACS DIVA software which was used for cell sorting are shown. Cells are gated on leucocytes (gate P1) and subsequently on singlets by FSC (gate P2) and SSC (gate P3). Gating of $CD62L^{hi}$ (FITC) and $CD4^+$ (PE-Cy7) (gate P4) selects naïve cells and by excluding $CD25^+$ (PE) and $CD44^{hi}$ (APC) (gate P5) non-activated cells are chosen.

5.3.1.2 NKT cells

For some experiments NKT cells were sorted from single cell suspensions of splenocytes. Cells were stained for CD8, CD11b, CD11c, B220 and DX5 with PE conjugated Abs and for NK1.1 with APC labeled Ab. The sorting gate was targeted on PE^- APC^+ cells to isolate NKT cells.

5.3.1.3 Dendritic cells

DCs were sorted either from BMDC culture or for some experiments from splenocytes. The cells were stained and sorted for CD11c and MHC class II. The target population was $CD11c^+$ and MHC class II intermediate/high. When sorting DCs from spleen it was necessary to sort the cells two times to achieve a purity >99%.

5.3.2 Analysis of cells

Cells were analyzed using either BD FACSCanto or BD LSR2. From every sample at least $1x10^4$ cells from *in vitro* proliferation experiments and $1x10^6$ cells from *in vivo* experiments were acquired. After spectral overlap compensation with the BD FACS Diva Software, FCS-files were exported and analyzed with FlowJo software from Tree Star.

5.4 Determination of cell numbers

To determine cell numbers in suspensions the electronic cell counter Z2 from Beckman Coulter was used. The cell suspensions were diluted 1:500 or 1:1000 in Isoton II. Using the Multisizer analyzer and Accucomb software, the cell population with a size between 5 µm and 16 µm of diameter was chosen and counted. Everything below 5 µm was debris and was not counted.

5.5 Propagation and titration of H5N1 influenza virus

Fertilized SPF hen eggs incubated for 10 days at 37°C at 50-70% humidity and regularly rotated. Virus stock was diluted in PBS with 1:10 serial dilutions from 10^{-2} to 10^{-4}. The eggs were candled and embryonated eggs at the same stage of development selected. For each virus dilution 5 eggs were infected by injection into the chorioallantoic cavity. The holes were sealed and eggs were incubated for 48 h and subsequently stored overnight at 4°C. Eggs were carefully opened and allantoic fluids were collected, pooled for each concentration and stored at 4°C. The virus titer was determined by hemagglutination test (HA-test). For this test 1:2 serial dilutions from allantoic fluids were performed in round bottom 96 well plates. To each well the same volume of standardized red blood cell (RBC) suspension was added and plates were gently vortexed. After 30-45 min at room temperature the highest dilution of

virus that caused complete hemagglutination was determined. The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination.

5.6 *In vitro* proliferation assay

5.6.1 Stimulation with antigen

For the in vitro proliferation assays, sorted naïve non-activated $CD4^+$ T cells from TRC transgenic mice were labeled with CFSE and co-cultured with BMDCs plus antigen for 4-5 days in the incubator. In some experiments, DCs sorted from spleens (sDCs) were used. For most experiments $1x10^5$ T cells were co-cultured with $1x10^4$ BMDCs and in some experiments also with $1x10^4$ NKT cells in round bottom 96-well plates. The used antigen dose ranged from 0.02-100,000 nM of OVA-peptide AA323-339 or HA-peptide AA110-120.

5.6.2 Stimulation with αCD3 and αCD28 antibodies

For obtaining $CD4^+$ T cells proliferation independent of antigens, αCD3 and αCD28 antibodies were used. In about half of the experiments spleen cells depleted from CD8 cells by FACS were used ($5x10^5$ cells per well of a 96-well plate with flat bottom). For the other part of experiments, sorted naïve non-activated $CD4^+$ T cells co-cultured with BMDCs were used, as described above (5.6.1). Since the used antibodies stimulate T cells directly by binding to the CD3 complex, activation of cells is independent from the Ag specificity of the TCR. With this method it is possible to stimulate cells from wild type animals (C57BL/6 and BALB/c) and from TCR transgenic mice (OTII and DO11.10). To measure the proliferation, CD8 depleted spleen cells or sorted $CD4^+$ T cells were labeled with CFSE after sorting. The cells were cultured in the incubator for 4-5 days in the presence of 1 µg/ml αCD28 Ab and αCD3 Ab titrated in a range of 0.03-64 µg/ml.

5.6.3 Culture conditions

For the *in vitro* proliferation assays different culture conditions were used. Beside the “normal” conditions (only complete RPMI), for some experiments Th17 polarizing conditions were necessary. These “Th17 inducing conditions” were achieved by

adding IL-6 (20 ng/ml) and TGFβ (1 ng/ml) to the cell culture. In some experiments neutralizing Abs against IL-2, IL-4 and IFNγ (all 10 µg/ml) were added to the culture.

5.6.4 Restimulation and staining for flow cytometry

After 4-5 days, plates from *in vitro* proliferation assays were centrifuged and the SN removed by a fast flip of the plate. To restimulate the cells, 100 µl of medium supplemented with ionomycin (1 µg/ml) and phorbol 12-myristate 13-acetate (PMA) (0.01 µg/ml) were added per well and cells were incubated for 4 h. For the last 2 h of the incubation 100 µl medium supplemented with ionomycin, PMA and brefeldin A. The brefeldin A was double concentrated so that the final concentration in each well was 5 µg/ml. After restimulation, cells were stained for viability and CD4 and fixated with PFA. Intracellular staining for IL-4, IL-17 and IFNγ was performed after permeabilization.

5.7 Adoptive transfer

For analyzing the T helper polarization *in vivo,* sorted naïve $CD4^+$ T cells from OTIIxThy1.1 double transgenic animals were adoptively transferred to normal C57BL/6 mice. Since the transferred T cells express Thy1.1 (CD90.1) and all T cells from normal C57BL/6 express Thy1.2 (CD90.2), the two cell populations can be distinguished by flow cytometry by staining for this marker. Recipient mice received $2x10^6$ CFSE labeled cells by tail vein injection and were immunized the following day with 0.4-625 µg OVA plus 25 µg LPS, either by intraperitoneal injection (i.p.) or by intra food pad injection (i.fp.). After 3-5 days the mice were sacrificed and spleens were taken. From mice, immunized i.p. before, additionally a peritoneum lavage (2 x 3 ml PBS) was performed, whereas from animals immunized i.fp. the draining popliteal LNs were isolated. The isolated cells were restimulated with ionomycin + PMA and brefeldin A was added, as above described (5.6.4).

After staining for CD3, CD4, CD90.1 and viability, cells were fixated with 2% PFA. For staining intracellular cytokines, cells were permeabilized and fluorochrome labeled Abs against IL-4, IL-17 and IFNγ were added, as described in 5.2.2. Read out was performed by flow cytometry.

5.8 Immunizations

5.8.1 Immunization strategy

5.8.1.1 Ovalbumin titrations

Groups of 3 or 5 six to eight weeks old C57BL/6 mice were immunized by either i.p. or s.c. route on day 0, 14 and 21 of the experiment. The animals were given different dosages of OVA ranging from 0.2 to 625 µg plus 20 or 25 µg LPS per mouse. Control groups received PBS, 20 or 25 µg LPS, or 25 µg OVA alone.
On day 42 mice were sacrificed and sampled.

5.8.1.2 H5N1 virosome titrations

For the combined immunization and challenge study, 6 groups of 15-16 six to eight weeks old BALB/c mice were immunized by i.n. route on day 0, 14 and 21 of the experiment. Each animal received 2.5 µg of c-di-AMP co-administered with 0.1, 0.5, 2.5 or 7.5 µg H5N1 virosomes diluted in 20 µl Ampuwa by application of 10 µl of solution to each nostril while anesthetized by isofluran inhalation. Control group mice received Ampuwa or 7.5 µg H5N1 virosomes alone. On day 42 five mice of each group were sacrificed and sampled. The remaining 10-11 mice per group were challenged with H5N1 virus, as described in section 5.8.2.

5.8.1.3 Immunizations including αGCPEG

Groups of 5 six to eight weeks old C57BL/6 or Jα281 knock out (KO) mice were immunized by i.n. route with 50 µg OVA co-administered with either 5 µg αGCPEG, 10 µg LPS, 1 µg BPPcysPEG, 10 µg CTB, 200 µg Curdlan, 20 µg CpG, 7.5 µg ISCOM or 2.5 µg c-di-GMP. Control animals received PBS or OVA alone. In most experiments animals were immunized on day 0, 14, 21 and sacrificed on day 42, whereas in experiments with Jα281 KO mice the animals were only immunized on day 0 and 14 and sacrificed on day 35.

5.8.2 H5N1 challenge of immunized mice

Mice immunized with H5N1 virosomes as described in section 5.8.1.2 were challenged with $5x10^5$ focus forming units of H5N1 virus in 50 µl of PBS by i.n. route

on day 49. For the next 6 days the animal weight was determined in the morning and animal health conditions were monitored in the morning and in the evening. Mice which lost 25% or more of starting bodyweight or were in bad physiological conditions were sacrificed and considered as killed by the infection. After 6 days all remaining animals were sacrificed.

5.8.3 Sampling of mice

The immunized animals were anesthetized with isofluran inhalation and 500 µl of blood were taken with glass capillaries from the retro-orbital plexus. Animals were sacrificed by CO_2 inhalation afterwards and spleens were isolated and processed as described in 5.1.1. To obtain serum the blood samples were incubated for 1 h at 37°C to coagulate, followed by incubation for 30 min at 4°C to enable the retraction of coagula. For separating serum from cellular blood components, tubes were centrifuged for 10 min at 7000 rpm. SNs were transferred to new tubes and stored at -20°C.

5.8.4 Enzyme linked immunospot assay (ELISpot)

For characterization of antigen specific cytokine producing spleen cells of immunized mice ELISpot assays were performed. The day before sampling, 96 well pates with hydrophobic high protein binding Immobilin-P-Membranes on the flat bottom were coated with capture antibodies against IL-4, IL-17 or IFNγ diluted in PBS overnight at 4°C. The next day, plates were washed and incubated for 2 h at RT with complete medium to block unspecific binding sites on the membrane with protein from the medium. For every sample, 11 wells with $5x10^5$ spleen cells per well were used. The first 5 wells were restimulated with 5 µg/ml of OVA or 2 µg/ml of H5N1 virosomes, whereas the next 5 wells were left unstimulated to determine unspecific cytokine production (background). The last well was stimulated with 5 µg/ml of concanavalin A (Con A) as positive control. Unused wells on each plate were filled with medium alone as negative controls. The plates were incubated at 37°C and 5% CO_2 for 24 (IFNγ) or 48 h (IL-4 and IL-17). To stop cytokine production, plates were washed with deionized water followed by washing with buffer (0.05 % Tween-20 in PBS). To detect captured cytokines on the membranes, corresponding biotinylated detection antibody diluted in buffer (10% FCS/PBS) was added and incubated for 2 h at room

temperature (RT). After washing with wash buffer, horseradish peroxidase (HRP) coupled to avidin was added and incubated for another hour at RT. The plates were then washed with wash buffer and subsequently with PBS. To visualize the captured cytokines, substrate solution was added and within the next 5 to 30 min red/brownish spots appeared by the HRP catalyzed reaction. The reaction was stopped by washing the plate with deionized water. Plates were dried overnight at RT in the dark and then scanned with the ImmunoSpot series 3A analyzer. The spots were counted by using the ImmunoSpot image analyzer software (version 3.2; Cellular Technology, Ltd.). Every spot in this assay represents one cell producing the captured cytokine. For every sample spot forming units (SPU) per $1x10^6$ spleen cells subtracted by the background from unstimulated cells were calculated. The results are presented as mean values +/- the standard error of the mean (SEM) and the subtracted background levels are given for each experiment. All antibodies for ELISpot were used according to the manufacturer's protocols supplied in the kits.

5.8.5 Proliferation assays based on ^{3}H-thymidine incorporation

Antigen specific proliferation of cells from immunized mice can be determined by measuring the incorporation of radioactive ^{3}H-thymidine into the DNA after antigen restimulation. Spleen cells from all animals per group were pooled and $5x10^5$ cells per well were seeded in 96 well plates. To restimulate the cells, either OVA was added to final concentrations of 10, 20, 30 and 40 µg/ml or H5N1 virosomes with final concentrations of 0.1, 1 and 2 µg/ml. As a positive control ConA was used at a final concentration of 5 µg/ml, whereas negative controls were cultured in medium alone. For all conditions quadruplicates were cultured in the incubator for 96 h. For the last 16-18 h, 1 µCi/well of ^{3}H-thymidine was added to the plates. Then, cells were harvested on filter mats with the cell harvester ICH-110 and dried in a microwave for 30 s. Melt-on scintillator sheets were melted on the filter forming a wax layer which acts as a solid scintillation material. The concentration of ^{3}H-thymidine was determined by measuring the radioactivity in counts per minute (cpm) using the γ-scintillation counter 1450 MicroBeta. Results were presented as mean values +/- the SEM.

5.8.6 Identification of T helper and polyfunctional $CD4^+$ T cells by flow cytometry

For 3 mice per group $2x10^7$ spleen cells per animal were cultured with either 2 µg/ml of H5N1 virosomes for restimulation or medium alone as control for 20-24 h. For the last 4 h, 5 µg/ml brefeldin A was added to every sample. Cells were stained for viability, CD3, CD4 and CD8. After fixation, cells were permeabilized and stained for IL-2, IL-4, IL-17, IFNγ and TNFα. At least $1x10^6$ cells per sample, viable at the time point of staining, were acquired by LSRII.

5.9 Statistics

Statistical analyses were performed with the GraphPad Prism 5 software. For multiple group comparisons, one-way ANOVA or two-way ANOVA were applied. For comparisons of independent groups, Student's *t* test or the Mann-Whitney test were performed. For comparisons of matched groups, paired Student's *t* test or Wilcoxon matched test were performed. In figures, n.s. indicates not significant; *** indicates $p < 0.001$; ** indicates $p < 0.01$; and * indicates $p < 0.05$.

6 Results

6.1 Influence of antigen dose on T helper polarization

6.1.1 Setup of the *in vitro* proliferation assay

To study the polarization of naïve $CD4^+$ T cells into different Th phenotypes a reliable and simple model had to be established. An *in vitro* proliferation assay with sorted naïve $CD4^+$ T cells from TCR transgenic mice and the corresponding Ag presented by sorted BMDCs fulfills these criteria. The workflow for this assay, as well as the following data acquisition and analysis are shown schematically in Fig. 9 and explained in detail in section 5.6. In a first set of experiments, Ag concentrations were defined, ranging from those not inducing proliferation at all to those triggering induction of full proliferative T cell responses.

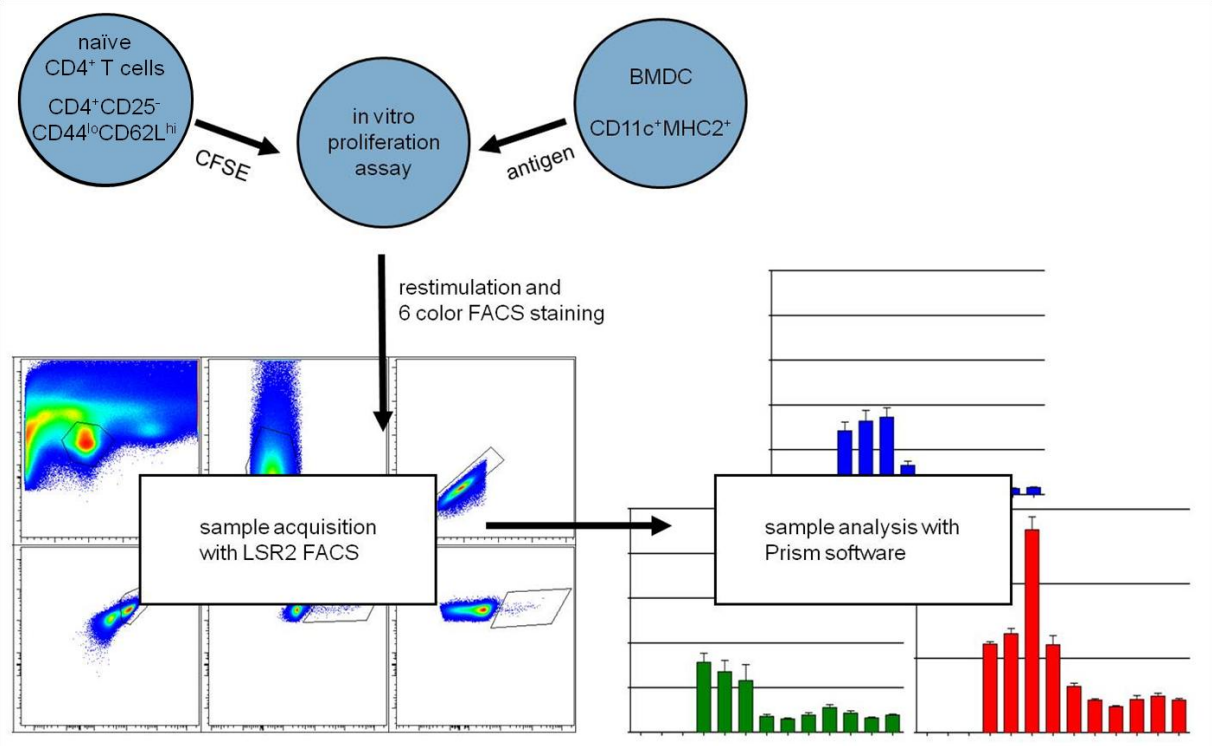

Fig. 9: Schematic workflow for the established *in vitro* proliferation assay. Sorted naïve $CD4^+$ T cells from DO11.10 or OTII mice were labeled with CFSE and co-cultured with sorted BMDCs in the presence of different Ag concentrations for 5 days. After restimulation with ionomycin and PMA cells were stained with fluorescent labeled Abs and acquired with a LSRII. Analysis of samples was performed using FlowJo and subsequently GraphPad software.

The strongest proliferation rates of OTII and DO11.10 $CD4^+$ T cells are normally achieved using OVA-peptide AA323-339 at concentrations around 500-1000 nM (0.89-1.78 µg/ml) [215]. The Ag dose for the presented experiments was limited to a concentration of 100000 nM due to the pH buffering capacity of the cell culture medium. Higher Ag concentrations resulted in a color change of medium, indicating acidic conditions which are not favorable for cell culture experiments. Thus, the Ag concentration of 100000 nM was used as starting point for a 1:3 serial dilution down to 1.7 nM. To measure the Ag dose dependent proliferative capacity of $CD4^+$ T cells, FACS sorted naïve $CD4^+$ T cells from OTII mice were labeled with CFSE and stimulated with different Ag concentrations in the presence of BMDCs. It was observed that T cell proliferation started at an Ag concentration between 5 to 15 nM, reaching the peak of proliferative capacity (84%) between 412 and 1235nM and persisted at this level for higher Ag doses (Fig. 10). Therefore the range of Ag concentrations from 100000 nM down to 1.7 nM (or 0.2 nM in some experiments) fulfills the before defined criteria and was used in the following experiments. The same Ag titration experiments performed with DO11.10 cells displayed comparable results (data not shown). Cells that were not stimulated with Ag served as controls and did not show any proliferation. In the subsequently performed *in vitro* proliferation assays divided $CD4^+$ T cells (as identified by CFSE dilution) that were viable at the time point of staining (identified by fixable live/dead stain) were analyzed for cytokine production.

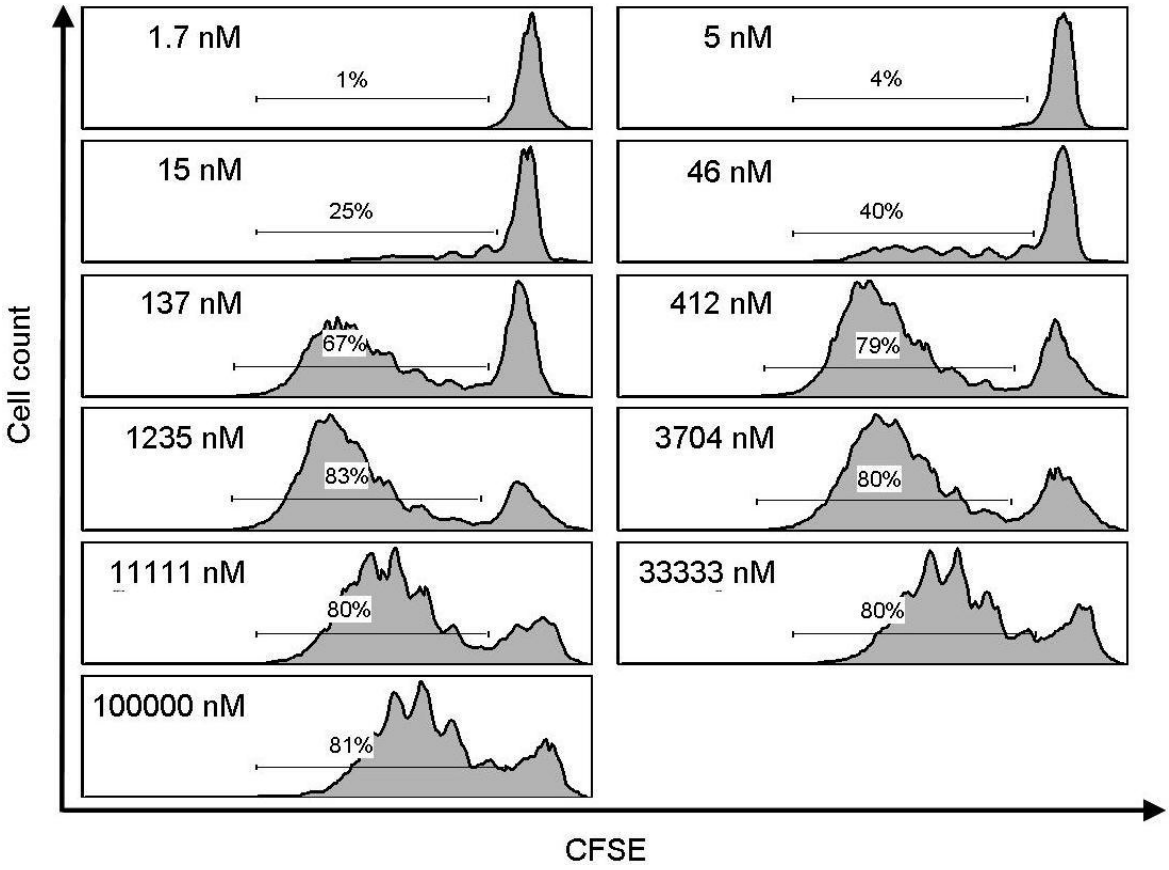

Fig. 10: Proliferation of OTII cells stimulated with different Ag concentrations. Histograms display the CFSE signal from OTII cells, which were co-cultured with BMDCs in the presence of the indicated OVA-peptide AA323-339 concentrations for five days. With every cell division, the CFSE signal strength is approximately halved. The shown percentages indicate the ratio of proliferated live $CD4^+$ T cells.

6.1.2 *In vitro* proliferation assay with OVA-peptide

6.1.2.1 Comparison of DO11.10 and OTII cells

Differences in Ag induced Th1/Th2 immune response are described when comparing C57BL/6 and BALB/c mice [62]. To investigate whether the animal background influences the conducted experiments and in particular Th17 polarization *in vitro*, the proliferation assays were performed in parallel with cells obtained from DO11.10 (BALB/c background) and OTII (C57BL/6 background) mice. In both transgenic mouse strains the TCRs expressed on $CD4^+$ T cells recognize the OVA-peptide AA323-339. To mimic the natural setting of Ag uptake and presentation to T cells, sorted BMDCs from BALB/c or C57BL/6 mice were pulsed for 3 h with different OVA-peptide concentrations and subsequently washed with medium. Ag pulsed DCs were co-cultured with sorted CFSE-labeled naïve $CD4^+$ T cells isolated from DO11.10 or OTII mice at a 1 to 10 ratio for 5 days. After restimulation with ionomycin and PMA,

cells were analyzed by FACS for IL-4 (Th2), IL-17 (Th17) and IFNγ (Th1) production (Fig. 11). DO11.10 cells showed an induction of Th2 and Th17 cells at low Ag doses (5-46 nM), whereas Th1 polarization was detected at slightly higher peptide concentrations (15-137 nM). Th1 phenotype represented the dominant T cell subset independently of the used Ag concentration. A complete different picture was observed in the differentiation of OTII cells. Here, IL-4 and IFNγ were produced at medium to high Ag concentrations (416-1235 nM) whereas Th17 polarization was detected at very high peptide doses (33333 and 100000 nM). Comparing cells derived from both strains, a higher number of IL-4 and IL-17 producing $CD4^+$ T cells were detected when cells from OTII mice were used, whereas polarization to IFNγ production was observed in higher frequencies when using cells from DO11.10 mice. Not only differences in Th induction were observed, but also in relation with dependence of Th17 polarization on the Ag dose. $CD4^+$ T cells sorted from DO11.10 mice differentiated to IL-17 producing cells in the presence of low peptide concentrations, whereas OTII cells were only polarized towards the Th17 phenotype when stimulated with high Ag doses. This observation pointed to a general difference between the used mouse strains and was further investigated in subsequently performed experiments.

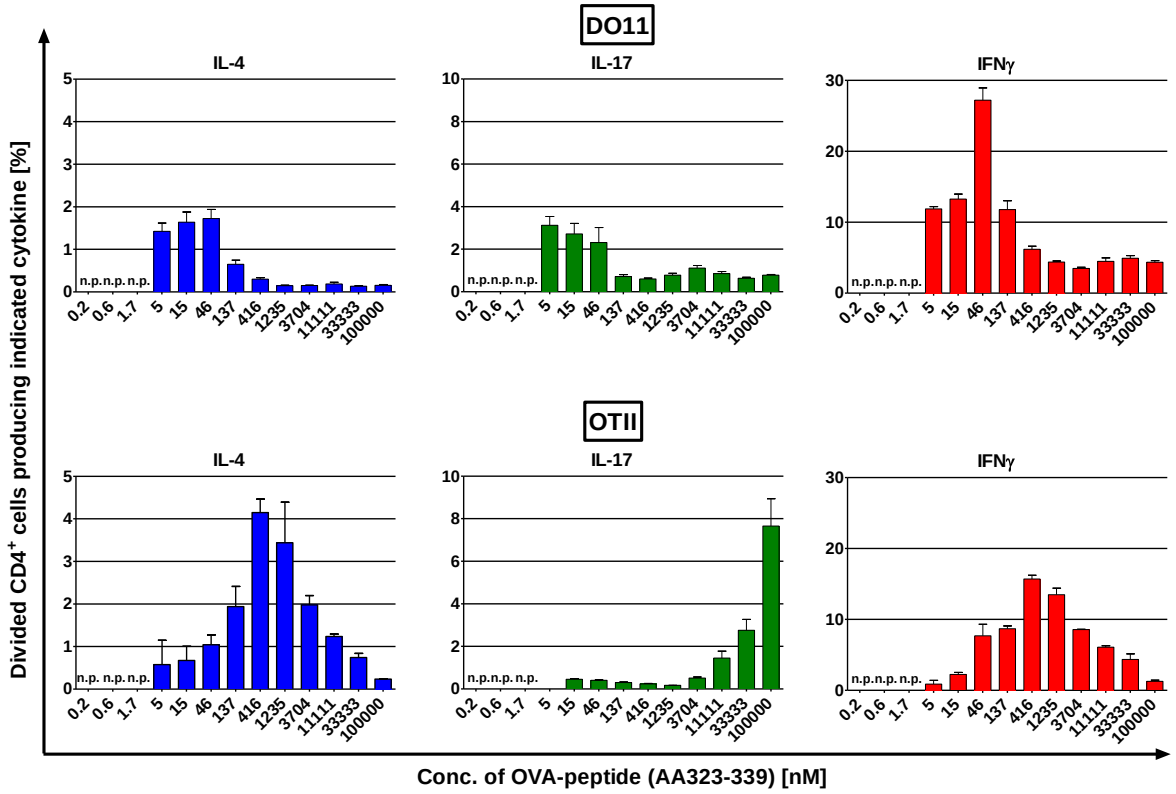

Fig. 11: Cytokine profiles of DO11.10 and OTII cells from *in vitro* proliferation assays with OVA-peptide. BMDCs were loaded for 3 h with Ag before co-cultured with naïve $CD4^+$ T cells for 5 days. Shown is the expression of the Th characteristic cytokines IL-4, IL-17 and IFNγ by viable, divided $CD4^+$ T cells. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation

In the above described experimental setting strongly fluctuating numbers of BMDCs per well were observed, suggesting an influence on the outcome of the assays. The washing process after the loading of DCs with Ag was identified as one reason for fluctuating cell numbers. Thus, the setup was slightly changed to guarantee constant and comparable conditions. For the following experiments the Ag was kept in culture for the whole incubation time of 5 days. The subsequently obtained results revealed T cell proliferation already at 0.6 nM, two dilution steps lower as compared to the previous experimental conditions (Fig. 4). The pattern of Th1 and Th2 polarization was quite similar between DO11.10 and OTII cells, and showed induction at low to medium peptide concentrations. Strikingly, there was a 5-fold and 2-fold increase of IL-4 and of IFNγ positive OTII cells, respectively, as compared to the previous experimental conditions. This increase was not observed when DO11.10 cells were used. The main differences were observed at the level of polarization of Th17 cells. The distribution of IL-17 producing OTII cells was comparable to the first method, whereas the DO11.10 cells showed a complete different behavior. Two peaks were observed for Th17 induction, the first one at the lowest Ag concentrations and the

second one at highest Ag concentrations. Remarkable was the detected increase of IL-17 positive OTII cells from about 8% to more than 21% in the presence of high peptide concentrations. Due to the modification of the experimental setup, not only constant starting cell numbers per well were obtained, but also a more homogeneous induction pattern of Th1 and Th2 cells for the two mouse strains. The previously observed differences in Th17 polarization were preserved. Thus, for all further *in vitro* proliferation assays, the Ag was kept in the culture for the whole incubation time.

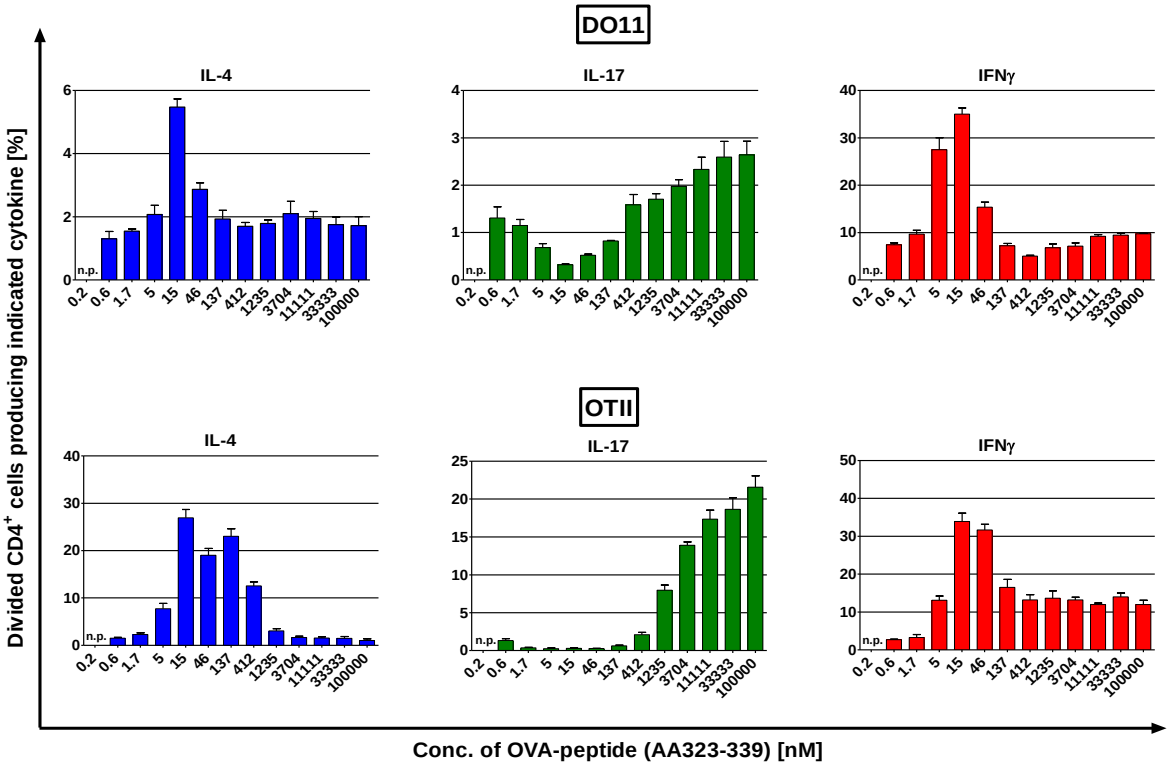

Fig. 12: Cytokine profiles of DO11.10 and OTII cells from *in vitro* proliferation assays with OVA-peptide. The Ag was present in the cultures during the whole of 5 days. Shown is the expression of the Th characteristic cytokines IL-4, IL-17 and IFNγ by viable and divided $CD4^+$ T cells. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

6.1.2.2 Th17 inducing conditions

Beside stimulation strength, the cytokine milieu is a major factor in Th differentiation. The effect of Th polarizing cytokines can also be Ag dose dependent. The cytokines IL-6 and TGFβ were described to induce Th17 polarization [65]. Therefore, it was investigated whether IL-6 and TGFβ influence Ag dose dependent Th17 differentiation in *in vitro* proliferation assays. The culture of cells in complete RPMI medium without any additional supplements is called “normal condition” and was compared to “Th 17 inducing conditions”, which were achieved by the addition of IL-6 and TGFβ to the cell cultures. The normal condition was included in all experiments as internal control, since fluctuations in the percentage of cytokine expressing cells were observed between different experiments, whereas the pattern of Th induction at different Ag doses was quite constant.

In the set of experiments performed with DO11.10 cells the results for IL-4 and IFNγ under normal conditions were comparable to the previous presented results (Fig. 13). However, a difference in Th17 polarization was detected under normal conditions, since the previously observed low dose peak for IL-17 was absent. Addition of IL-6 and TGFβ completely blocked the induction of Th2 cells and clearly reduced the Th1 polarization at all tested peptide concentrations. Regarding IL-17, the low dose peak as well as the high dose peak were detected under Th17 inducing conditions, and the overall percentage of Th17 cells was increased at all Ag concentrations.

The same set of experiments was performed with sorted naïve $CD4^+$ T cells derived from OTII mice (Fig. 14). Under normal conditions Th1 and Th2 cells were induced at low to medium Ag doses, whereas cells were polarized towards the Th17 phenotype at the highest peptide concentrations. The presence of polarizing cytokines in the culture led to almost complete blockage of Th1 and Th2 polarization, and the percentage of IL-17 producing cells was strongly increased. Especially at high peptide doses high numbers of IL-17 producing cells were observed (*i.e.* approximately every second cell differentiated to the Th17 phenotype).

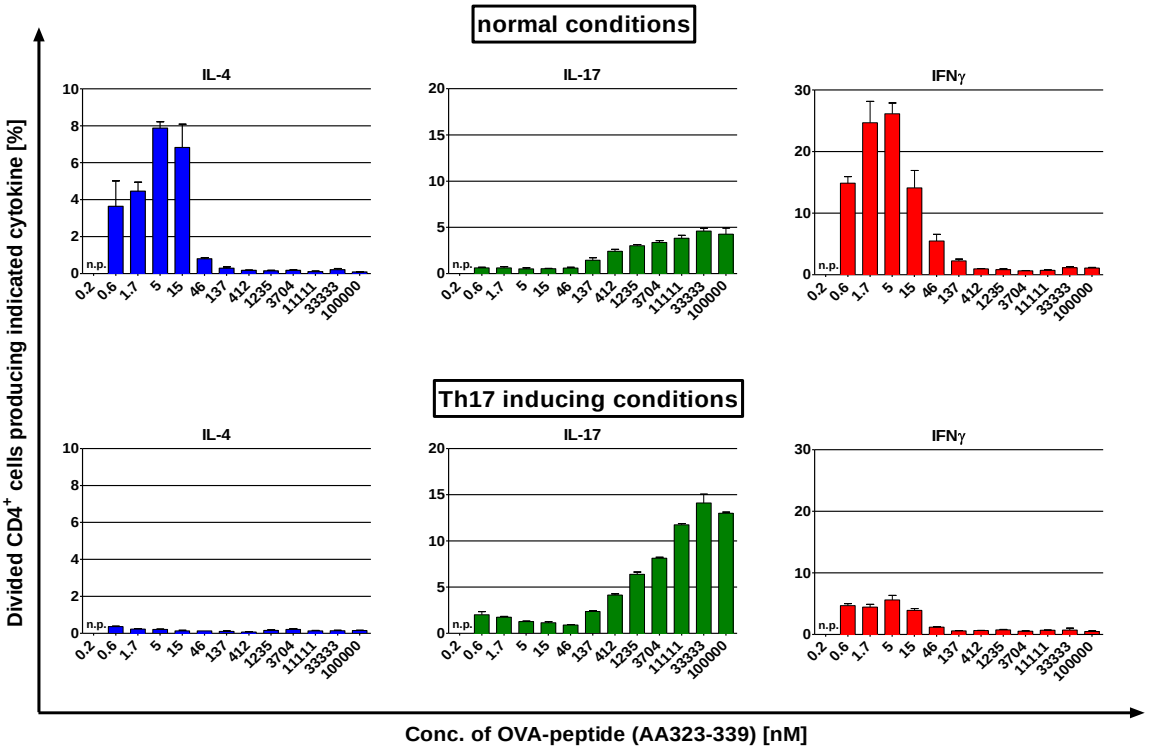

Fig. 13: Cytokine profiles of DO11.10 cells from *in vitro* proliferation assays with OVA-peptide. Naïve CD4$^+$ T cells sorted from DO11.10 mice were cultured under normal and Th17 inducing (IL-6 + TGFβ) conditions. Shown is the expression of the Th characteristic cytokines IL-4, IL-17 and IFNγ by viable and divided CD4$^+$ T cells after 5 days in culture. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

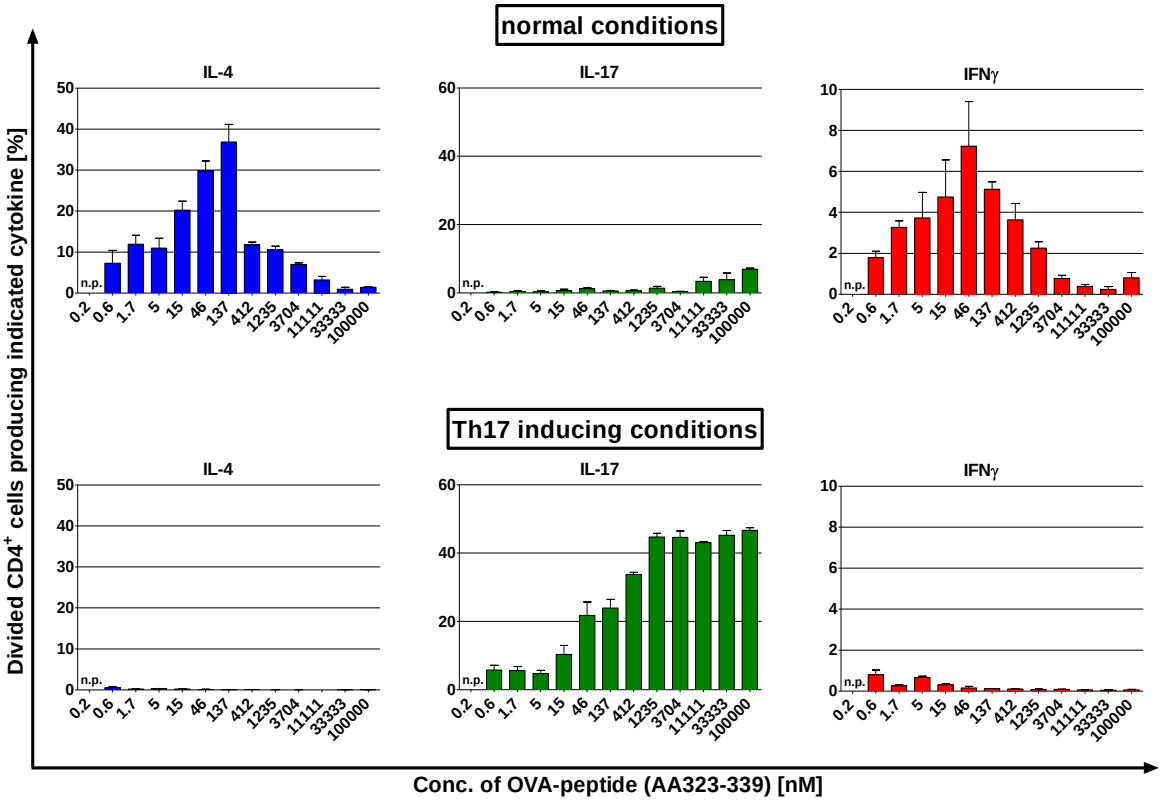

Fig. 14: Cytokine profiles of OTII cells from *in vitro* proliferation assays with OVA-peptide. Naïve CD4$^+$ T cells sorted from OTII mice were cultured under normal and Th17 inducing (IL-6 + TGFβ) conditions. Shown is the expression of the Th characteristic cytokines IL-4, IL-17 and IFNγ by viable and divided CD4$^+$ T cells after 5 days in culture. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

6.1.2.3 Neutralization of blocking cytokines

Published studies demonstrated that the Th polarization is not only affected by IL-6 and TGFβ but also by IL-2, IFNγ and IL-4 (see sections 2.1.3 and 2.1.4). To study the effect of these three cytokines on Th polarization in combination with different Ag doses, neutralizing Abs against IL-2 (αIL-2), IL-4 (αIL-4) and IFNγ (αIFNγ) were added to the cultures, either alone or in combinations. The experiments were performed in parallel with cells sorted from DO11.10 and OTII mice.

The addition of αIL-2 to DO11.10 cells led to a reduction of IL-4 producing cells, as compared to standard conditions (Fig. 15). In contrast, neutralizing Abs against IFNγ led to increased Th2 polarization at very low peptide concentrations. However, this increase was not observed when αIFNγ was added in combination with αIL-4 or αIL-2 and αIL-4. Instead, these combinations led to a reduced percentage of IL-4 positive cells. In assays with OTII cells, Th2 induction was observed at high peptide concentrations (Fig. 16). The neutralization of secreted IL-2, either alone or in combination with αIL-4 and αIFNγ, completely blocked Th2 polarization. As compared to DO11.10 cells, only a small increase in Th2 cell frequencies was detected when IFNγ was neutralized.

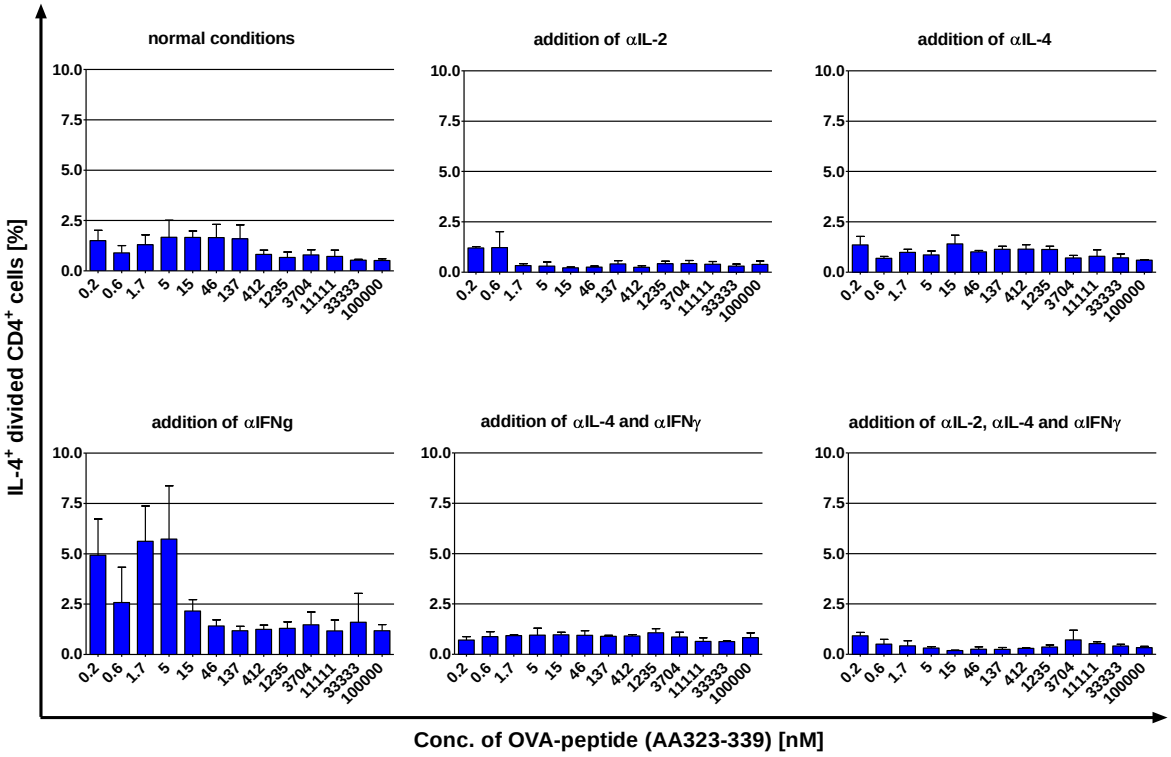

Fig. 15: IL-4 production of DO11.10 cells in the presence of different neutralizing Abs. Shown is the expression of the Th2 characteristic cytokine IL-4 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

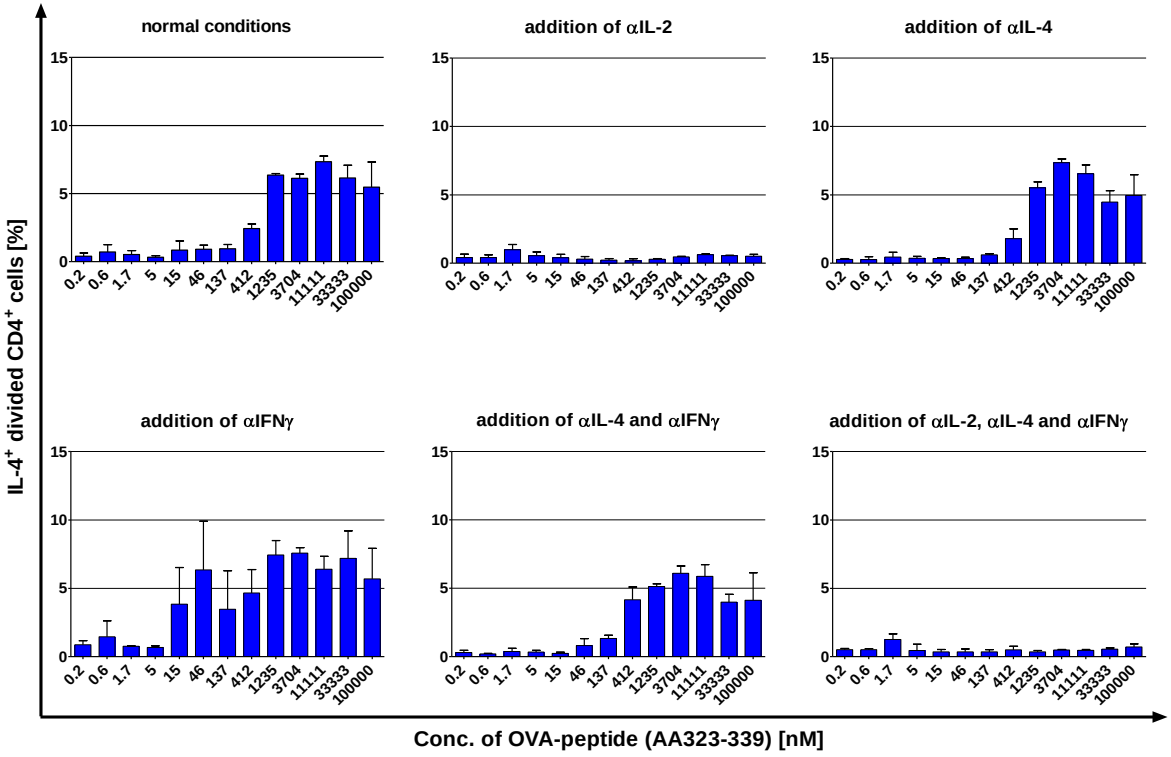

Fig. 16: IL-4 production of OTII cells in the presence of different neutralizing Abs. Shown is the expression of the Th2 characteristic cytokine IL-4 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

The polarization towards the Th17 phenotype in DO11.10 was positively influenced by the addition of neutralizing Abs, especially in the presence of αIFNγ (Fig. 17).The combinations of two or all three Abs led to a huge increase of IL-17 producing cells. The strongest increase was observed at high peptide concentrations. Due to this dose dependent increase of Th17 polarization, the peak of IL-17 producing cells which was observed under normal conditions at low doses, was less characteristic under neutralizing conditions. Also in the experiments performed with OTII cells, the addition of neutralizing Abs influenced Th17 induction (Fig. 18). As in all previous presented experiments with OTII cells, Th17 polarization was exclusively detected at high Ag doses. In the experiments with neutralizing Abs, only the addition of αIL-2 alone or in combination with αIL-4 and αIFNγ led to an almost 3-fold increase of IL-17 producing cells in the presence of high peptide concentrations.

Th1 cells were detected in DO11.10 mice at relatively low Ag concentrations (Fig. 19). The addition of αIL-2, the combination of αIL-4 and αIFNγ and especially the addition of all three Abs blocked the induction of IFNγ producing cells. In experiments performed with OTII cells a Th1 induction was observed at medium Ag doses (Fig. 20). The combined neutralization of IL-4 and IFNγ led to a 50% decrease of IFNγ producing cells. However, the strongest effect was observed by adding αIL-2 or the combination of all three neutralizing Abs. Under these conditions OTII cells were hardly polarized towards the Th1 phenotype.

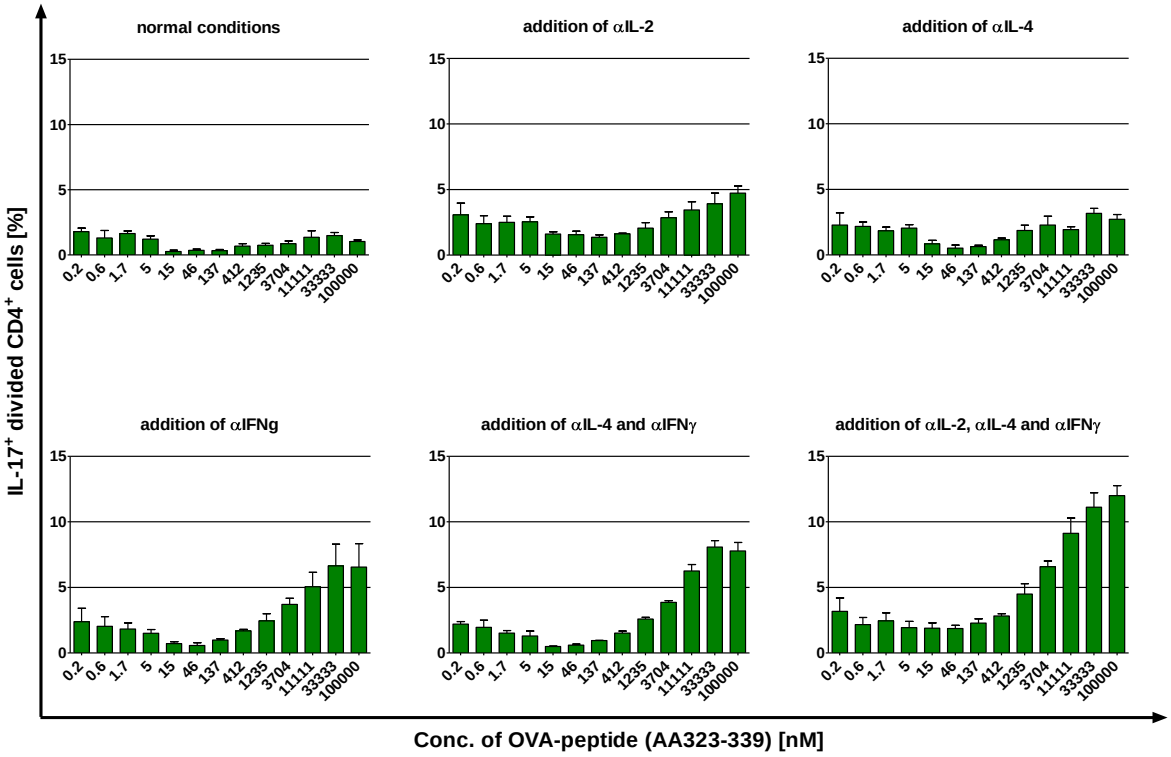

Fig. 17: IL-17 production of DO11.10 cells in the presence of different neutralizing Abs. Shown is the expression of the Th17 characteristic cytokine IL-17 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

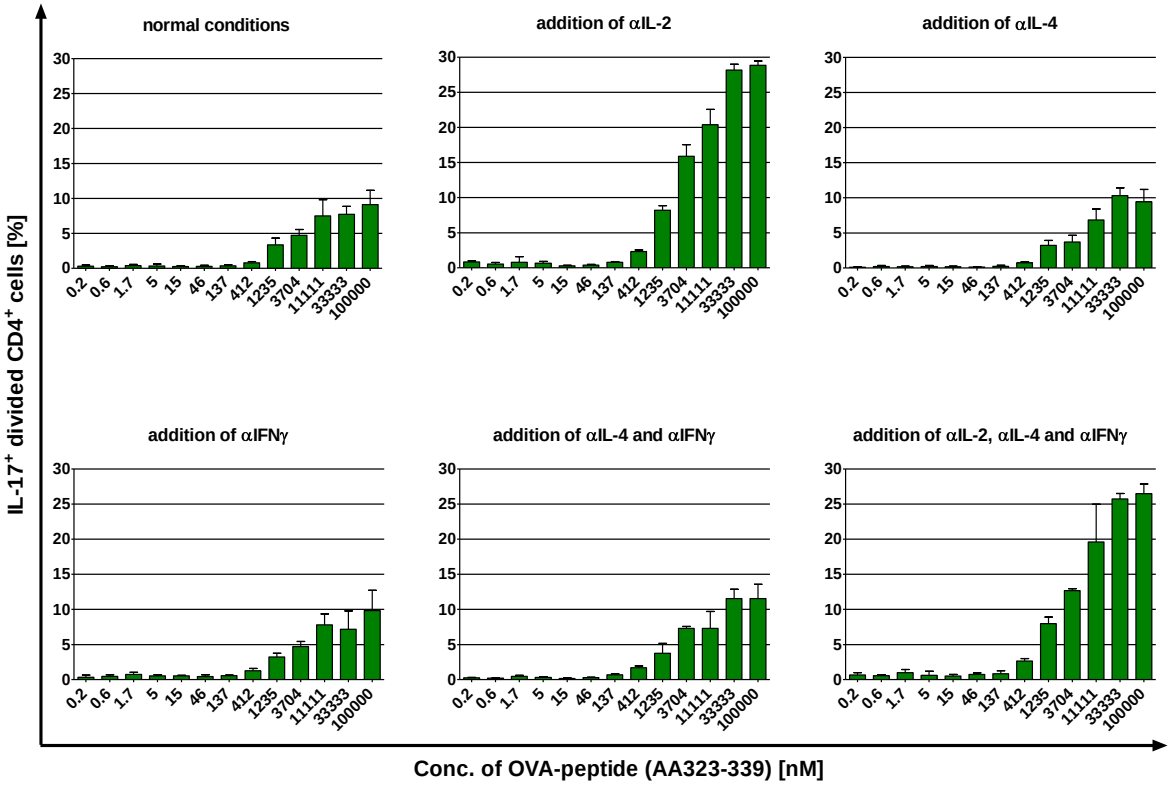

Fig. 18: IL-17 production of OTII cells in the presence of different neutralizing Abs. Shown is the expression of the Th17 characteristic cytokine IL-17 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

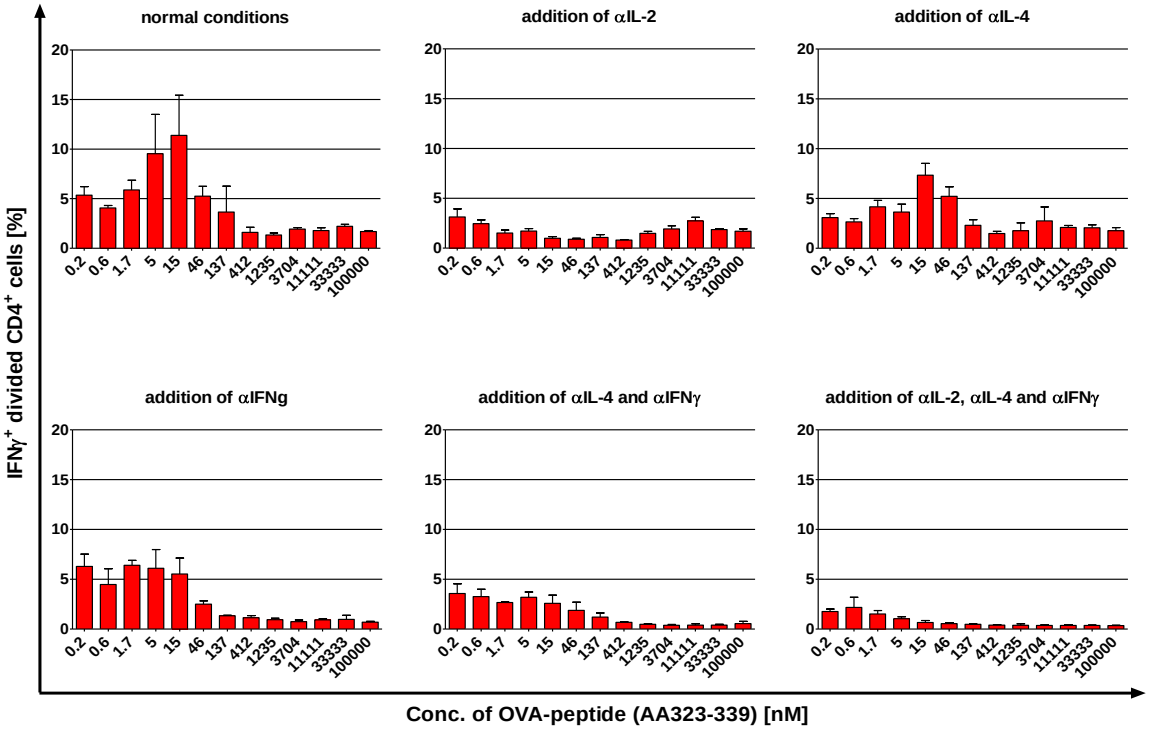

Fig. 19: IFNγ production of DO11.10 cells in the presence of different neutralizing Abs. Shown is the expression of the Th1 characteristic cytokine IFNγ by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

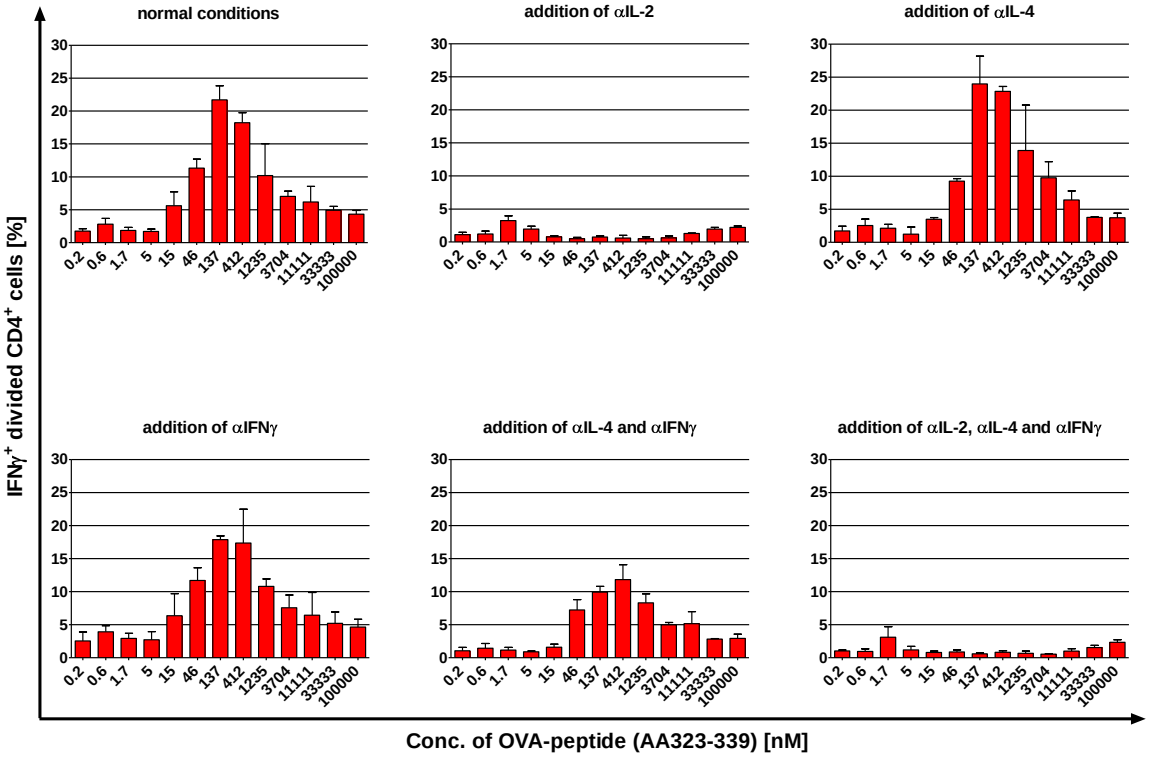

Fig. 20: IFNγ production of OTII cells in the presence of different neutralizing Abs. Shown is the expression of the Th1 characteristic cytokine IFNγ by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard culture conditions or in the presence of blocking Abs against IL-2, IL-4 and IFNγ alone or in combinations. Each bar represents the mean of three samples plus SEM.

In conclusion, for both mouse strains the strongest effects on Th polarization were observed when all three neutralizing Abs were added together to the *in vitro* proliferation assays. The induction of Th17 cells was strongly increased, whereas Th1 and Th2 subsets were almost absent in the presence of the combined neutralizing Abs.

6.1.2.4 Combination of Th17 inducing conditions and neutralization Abs

The previous results raised a new question, namely, whether Th17 induction follows the same Ag dose dependent pattern under highly polarizing conditions as observed before. Thus, in the following experiments cells obtained from DO11.10 and OTII mice were cultured under conditions in which combinations of all three neutralizing Abs together with Th17 polarizing cytokines were evaluated.

In assays performed with DO11.10 cells under normal conditions, an induction of IL-4 producing cells was observed at the lowest Ag dose and at high Ag dose (Fig. 21). Highest numbers of IL-17 producing cells were detected at the quite low peptide concentration of 15 nM, whereas the induction of Th1 cells was observed at medium Ag dosages. The combination of Th17 culture conditions and the addition of neutralizing Abs almost completely blocked Th1 and Th2 differentiation and only few IL-4 producing cells were present in samples with the lowest Ag concentrations. The detection of Th17 cells was considerably increased at Ag doses of 416 nM and higher, resulting in more than 40% of Th17 cells at the two highest peptide concentrations. However, this increase was not observed at low Ag doses. In fact, the low dose peak of Th17 cells, which was observed in the previous experiments with DO11.10 mice under Th17 inducing conditions or addition of neutralizing Abs alone, was absent.

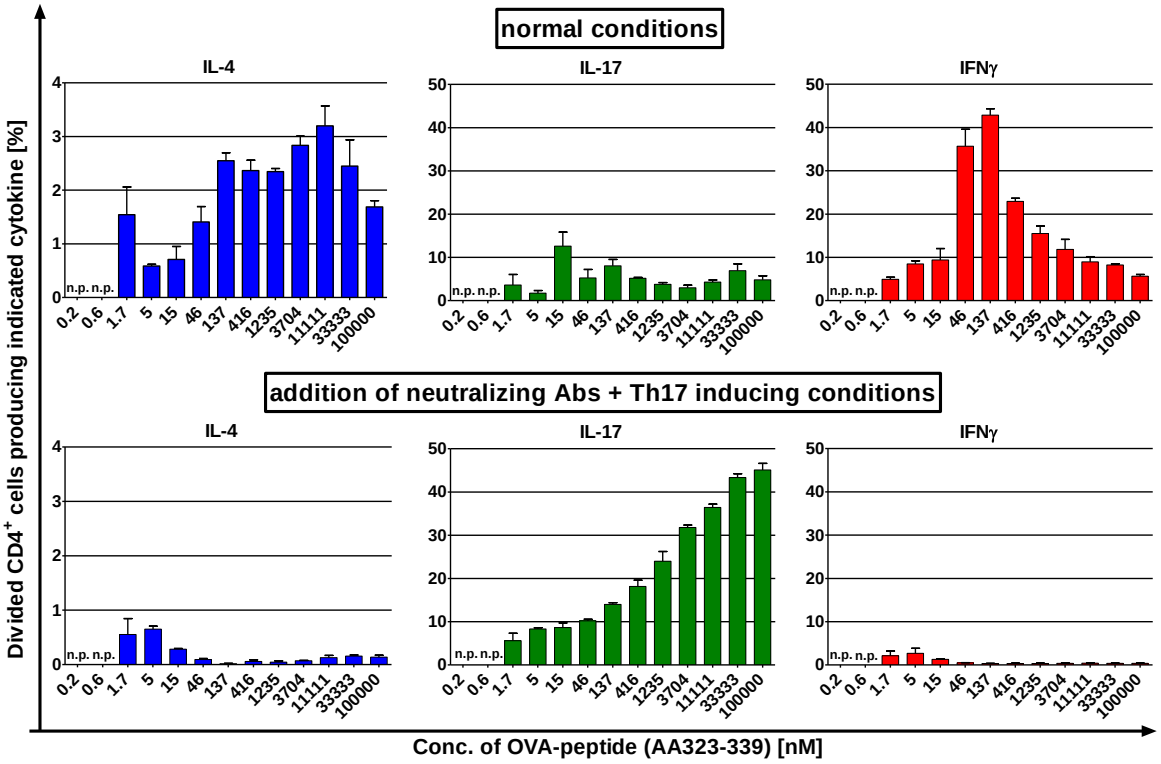

Fig. 21: Cytokine profiles of DO11.10 cells under Th17 polarizing conditions in the presence of neutralizing Abs. Shown are the Th cytokine profiles of viable and divided $CD4^+$ DO11.10 cells from *in vitro* proliferation assays with different OVA-peptide doses. Cells were either cultured under normal conditions or under Th17 inducing conditions in the presence of neutralizing Abs against IL-2, IL-4 and IFNγ. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

Proliferation assays with OTII cells were performed under the same conditions as for DO11.10 cells. Under normal conditions, the differentiation towards Th2 cells was very weak with less than 0.5% of $CD4^+$ T cells producing IL-4 (Fig. 22). IL-17 producing cells were instead detected in high numbers with rising Ag dose, with a clear trend of Th17 induction at highest Ag doses. High numbers of Th1 cells were present in the samples stimulated with medium peptide concentrations (*i.e.* around 137 nM). Surprisingly, addition of neutralizing Abs under Th17 inducing conditions resulted in an increase of Th2 polarization at the lowest Ag doses. In this context, it has to be stated that the general level of IL-4 producing cells was quite low as compared to previous experiments. As expected, these culture conditions boosted polarization of IL-17 producing cells starting from 416 nM OVA-peptide, where a maximum of 60% Th17 polarization was reached. In contrast, Th1 cells were almost absent under these culture conditions.

To conclude, in both TCR transgenic mouse strains the combination of Th17 inducing conditions and neutralizing Abs led to further increased Th17 polarization, especially at high Ag concentrations. Under these highly Th17 polarizing conditions, the low dose peak of IL-17 producing cells was not detected in DO11.10 cultures.

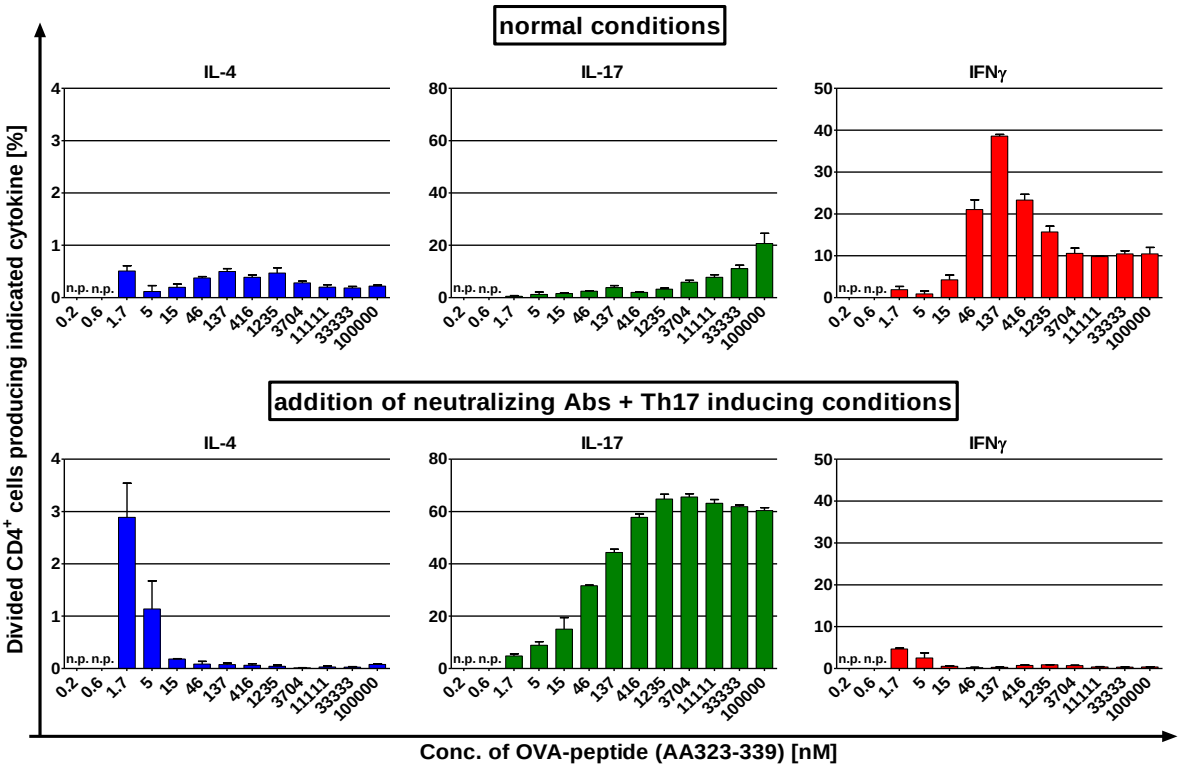

Fig. 22: Cytokine profiles of OTII cells under Th17 polarizing conditions in the presence of neutralizing Abs. Shown are the Th cytokine profiles of viable and divided $CD4^+$ OTII cells from *in vitro* proliferation assays with different OVA-peptide doses. Cells were either cultured under normal conditions or under Th17 inducing conditions in the presence of neutralizing Abs against IL-2, IL-4 and IFNγ. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

6.1.2.5 Neutralization of IFNβ

Published studies demonstrated that also type 1 IFNs can influence Th differentiation (see sections 2.1.3 and 2.1.4). Application of IFNβ is a common treatment of MS in humans [216], and was reported to reduce IL-17 levels in the EAE mouse model [217]. Thus, the effect of IFNβ on antigen dose dependent Th polarization was investigated by the addition of neutralizing Ab to the established *in vitro* proliferation assays with TCR transgenic $CD4^+$ T cells. The experiments were carried out with DO11.10 cells under normal and Th17 inducing conditions.

The highest numbers of Th2 cells were detected under normal culture conditions at peptide concentrations around 5 nM and the abundance of the IL-4 producing cells was halved by IFNβ neutralization in the cultures (Fig. 23). Th17 inducing culture conditions resulted in an almost complete blockage of the Th2 phenotype, regardless of the addition of αIFNβ Ab.

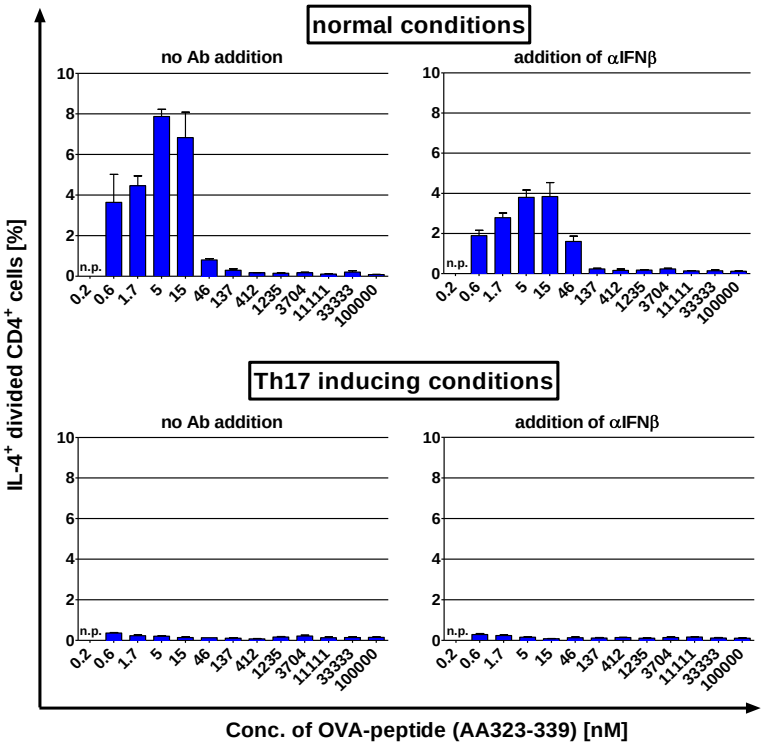

Fig. 23: IL-4 production of DO11.10 cells in the presence of αIFNβ under normal and Th17 inducing conditions. Shown is the expression of the Th2 characteristic cytokine IL-4 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions or in the presence of blocking Abs against IFNβ. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

IL-17 producing cells were induced under normal conditions at high and very high peptide concentrations. Neutralization of IFNβ resulted in no significant changes with respect to the polarization towards Th17 phenotype (Fig. 24). Worth to mention is the lack of the low dose Th17 peak in this experiment performed under normal conditions. The addition of IL-6 and TGFβ increased the induction of IL-17 producing cells at very low, high and especially at very high peptide concentrations. Under these Th17 inducing conditions, the low dose Th17 peak was restored. However, also under these experimental conditions the addition of neutralizing Abs did not result in any significant effect.

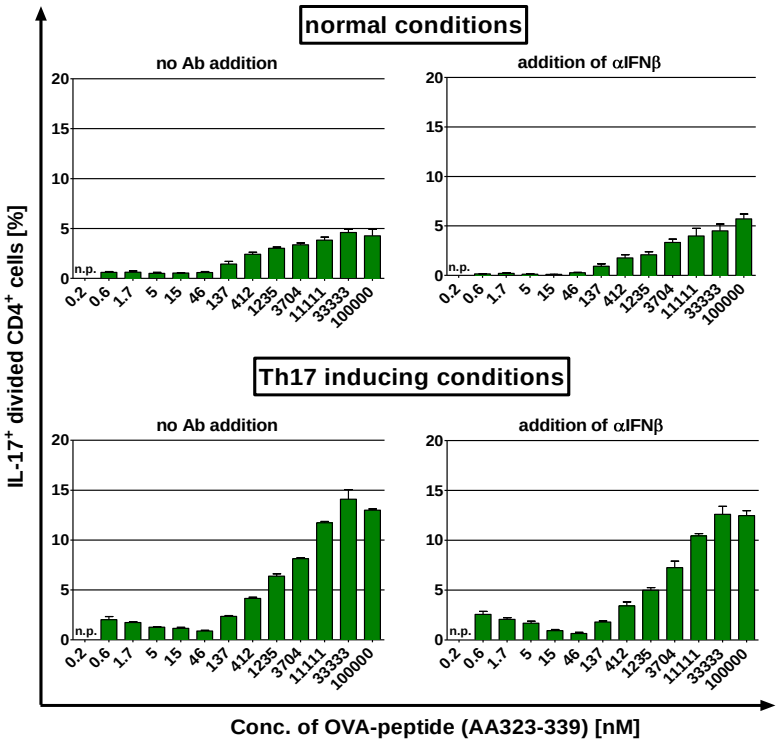

Fig. 24: IL-17 production of DO11.10 cells in the presence of αIFNβ under normal and Th17 inducing conditions. Shown is the expression of the Th17 characteristic cytokine IL-17 by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions or in the presence of blocking Abs against IFNβ. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

The induction of Th1 cells was highest at lower Ag doses around 5 nM (Fig. 25). As observed in the previous experiments, Th17 inducing conditions blocked Th1 polarization. In this experiment the abundance of IFNγ producing cells was reduced by the factor 5. Addition of αIFNβ Ab did not influence Th1 polarization, neither under normal nor under Th17 polarizing culture conditions.

Another way to visualize the results from this experiment is presented in Fig. 26. Here, instead of displaying the percentages of the viable and divided $CD4^+$ T cells that produce Th cytokines, the fraction of Th subsets for each Ag concentration was calculated. This representation of the data clearly demonstrated the dominance of Th17 cells at high and very high peptide concentrations under all performed culture conditions. As shown before, the increased induction of IL-17 producing cells at low Ag doses also resulted in a regime shift of Th subsets (see the two lower panels of Fig. 26).

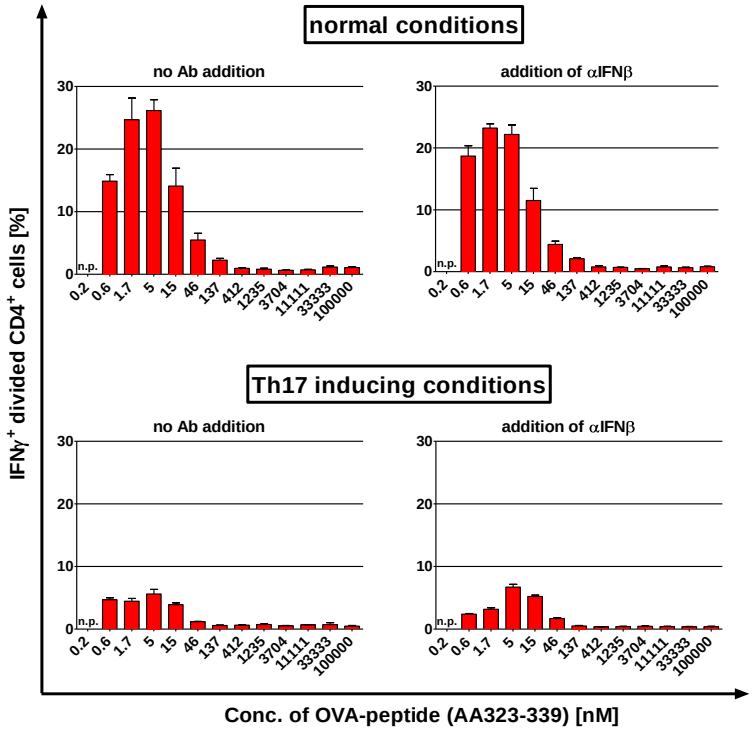

Fig. 25: IFNγ production of DO11.10 cells in the presence of αIFNβ under normal and Th17 inducing conditions. Shown is the expression of the Th1 characteristic cytokine IFNγ by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions or in the presence of blocking Abs against IFNβ. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

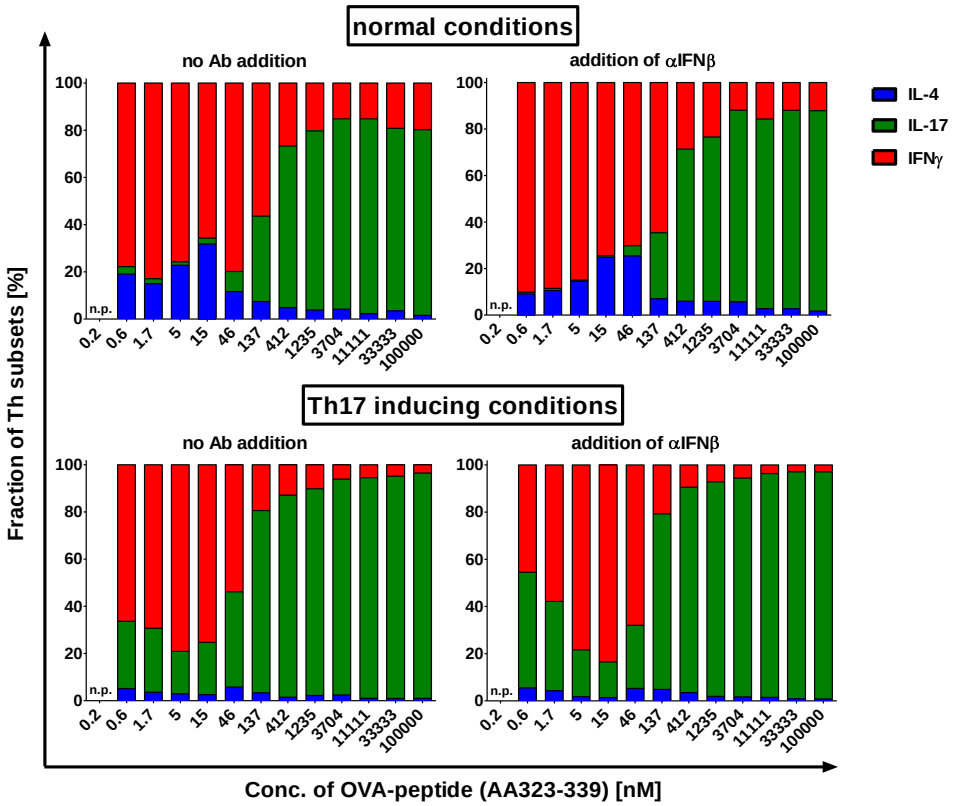

Fig. 26: Fractions of Th subsets under normal or Th17 inducing conditions in the presence of αIFNβ. For each peptide concentration the fractions of Th subsets from mean values of measured Th cytokine producing cells were calculated. Mean values were calculated from three samples. n.p.= no proliferation.

Taken together, the obtained results demonstrated that neutralization of IFNβ did not result in changes of Th polarization at all tested Ag concentrations and culture conditions, as observed by consistent production of IL-4, IL-17 and IFNγ by $CD4^+$ T cells.

6.1.2.6 Influence of DC to T cell ratio

The strength of stimulation is not only determined by the concentration of Ag, but also the number of DCs in the assay could have an effect on Th polarization. This effect is explained by differences in the available number of MHC class II molecules loaded with the Ag, as well as the degree of co-stimulation to T cells. Thus, the influence of the ratio between DCs and T cells on Ag dose dependent Th polarization was investigated by adding different amounts of sorted BMDCs to the *in vitro* proliferation assays. In all the experiments presented before the ratio between DCs and TCs was 1 to 10. Addition of less DCs per well resulted in a dramatic reduction of the proliferation rates. Especially at low peptide concentrations, the T cells neither

proliferated nor differentiated towards Th cells. An enhanced DC number led to slightly increased T cell proliferation and Th cytokine secretion. In the following experiments with OTII cells, the amount of DCs was increased by the factor 2 or 4 and compared to the standard ratio of 1 to 10 under both normal and Th17 inducing conditions.

The number of Th2 cells under normal conditions was very high as compared to previous experiments and a peak at 137 nM of OVA-peptide at the 1 to 10 ratio (Fig. 27). With increasing numbers of DCs in the culture, the polarization to Th2 phenotype was further increased, and the induction peak shifted to lower Ag concentrations. Th17 inducing conditions blocked the appearance of IL-4 producing cells almost completely at all DC to T cell ratios. Th17 polarization was induced at highest Ag concentrations under normal conditions (Fig. 28). The addition of more DCs to the culture clearly increased the percentage of IL-17 producing cells and shifted the start of Th17 induction to lower peptide concentrations. Under Th17 inducing conditions the percentage of Th17 cells multiplied by 4 at the 1 to 10 ratio and the induction started already at medium Ag doses and reached a maximum at higher Ag doses. The increase of DCs under these conditions did not change the Th17 profile. IFNγ producing cells were detected especially at medium doses around 46 nM under normal conditions (Fig. 29). In contrast to Th2 and Th17 cells, the dose dependent peak of Th1 cells was not shifted by the increased frequency of DCs in the culture. However, the percentage of Th1 cells was decreased when more DCs were present. As expected, the addition of Th17 polarizing cytokines blocked the induction of IFNγ producing cells. The different DC to T cell ratios did not influence this effect.

To summarize, the addition of higher DCs numbers to the cultures increased Th2 and Th17 induction under normal culture conditions but not when Th17 polarizing cytokines were present.

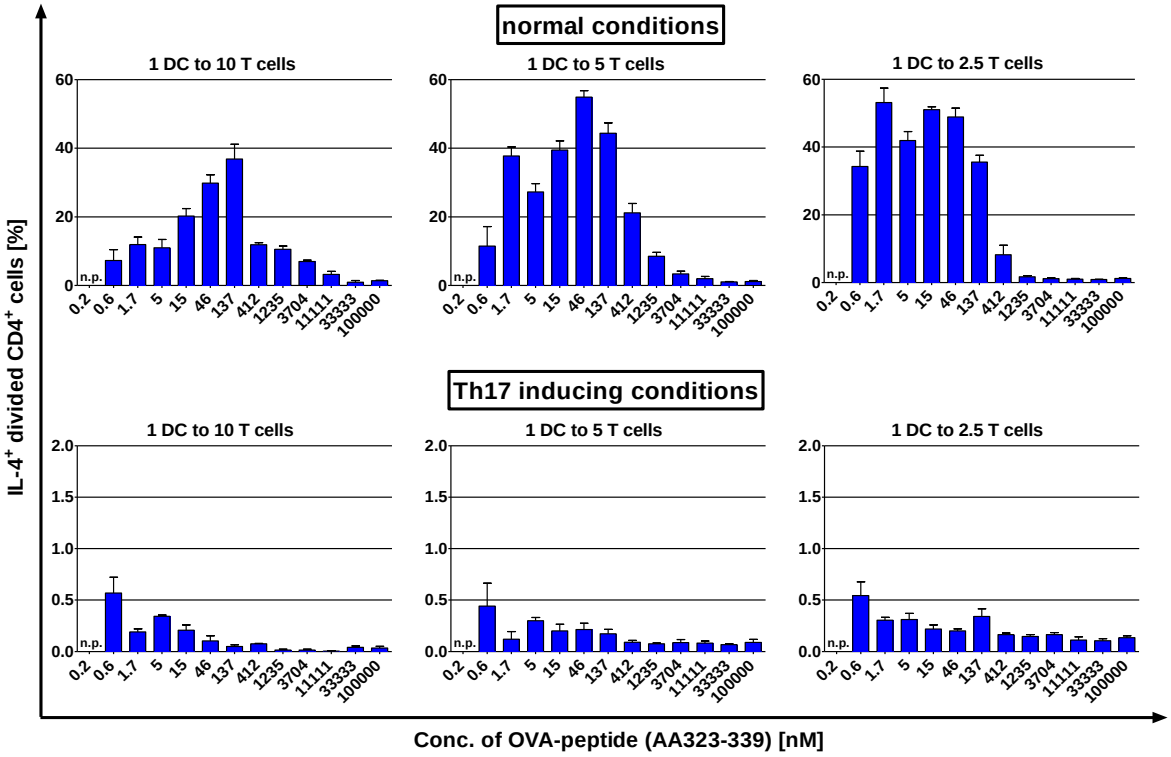

Fig. 27: IL-4 production of OTII cells cultured with DCs at different ratios. Shown is the expression of the Th2 characteristic cytokine IL-4 by viable and divided CD4$^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions at DC to T cell ratios of 1 to 10, 1 to 5 or 1 to 2.5. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

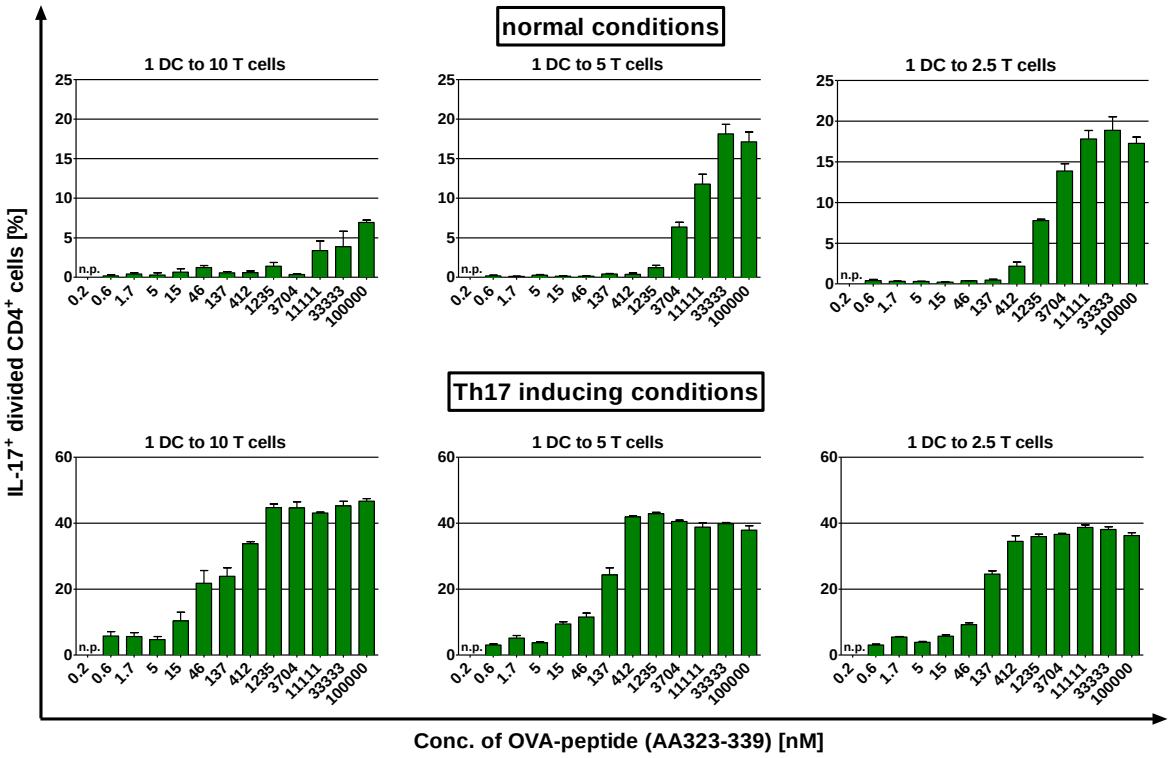

Fig. 28: IL-17 production of OTII cells cultured with DCs at different ratios. Shown is the expression of the Th17 characteristic cytokine IL-17 by viable and divided CD4$^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions at DC to T cell ratios of 1 to 10, 1 to 5 or 1 to 2.5. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

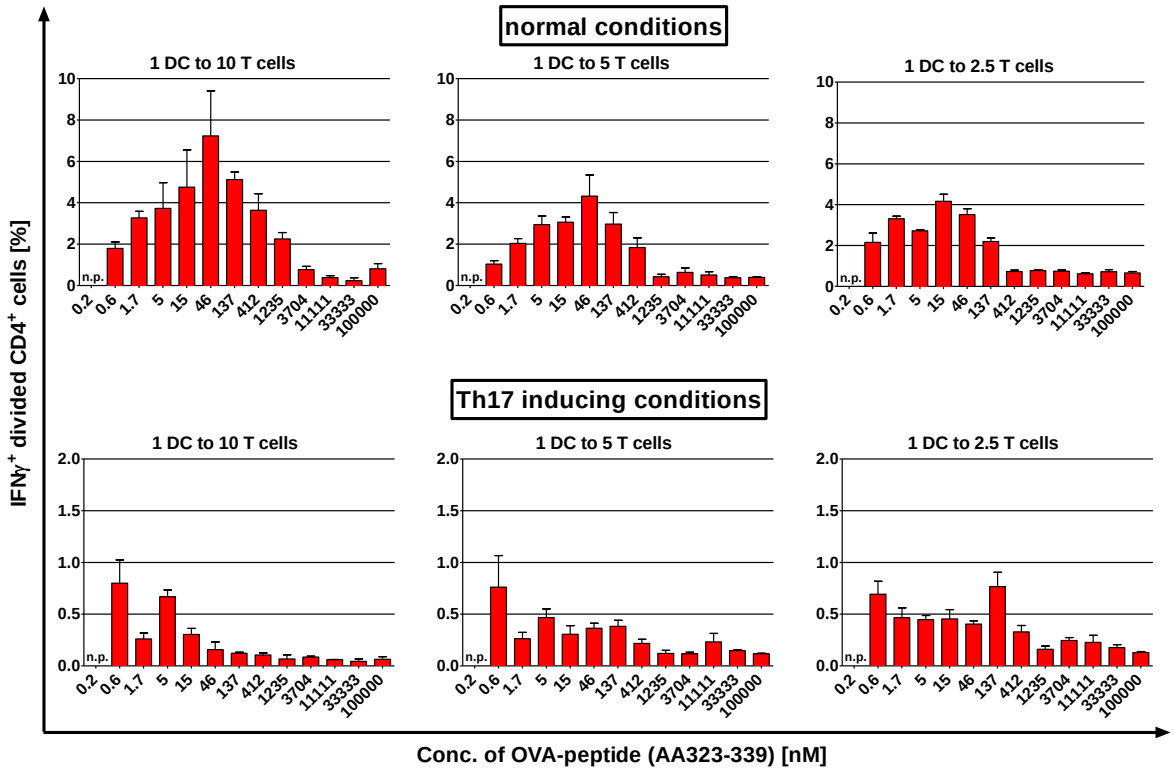

Fig. 29: IFNγ production of OTII cells cultured with DCs at different ratios. Shown is the expression of the Th1 characteristic cytokine IFNγ by viable and divided $CD4^+$ T cells stimulated with OVA-peptide under standard or Th17 inducing culture conditions at DC to T cell ratios of 1 to 10, 1 to 5 or 1 to 2.5. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

6.1.3 *In vitro* proliferation assay with HA-peptide

In the majority of the presented experiments a difference between DO11.10 and OTII mice in the Ag dose dependent Th17 induction was observed. DO11.10 cells displayed Th17 polarization either at very low Ag doses or at very low and very high doses, whereas cells from OTII mice were polarized towards Th17 phenotype only in the presence of high and very high Ag doses. They never differentiated in significant numbers to IL-17 producing cells at very low or low peptide concentrations. The following experiments were performed to reveal the reason for this unexpected difference between the two mouse strains. As a first step the Ag dose dependent induction of Th cells was investigated in a third strain, to compare if the results resemble either DO11.10 or OTII animals. For this experiment the TCR-HA transgenic mouse strain on BALB/c background featuring $CD4^+$ T cells specific for the HA-peptide AA110-120 from influenza virus PR8 was chosen. To assure similar conditions as in the proliferation assays presented before, the same sorting parameters, culture conditions and peptide concentrations were used. Th2 induction

was observed at low and medium Ag doses and the addition of Th17 polarizing cytokines resulted in decreased IL-4 producing cells (Fig. 30). Polarization towards Th17 cells was detected at very low and low peptide concentrations. A 3-fold increase was observed under Th17 inducing conditions at these Ag doses. Th1 cells were present in all samples at comparable amounts (between 6 and 9%), with the highest values at 100000 nM of the Ag. Th17 polarizing conditions again reduced the induction of IFNγ producing cells at all Ag doses, especially at high concentrations.

In summary, the cytokine profiles were more similar to those from DO11.10 cells than to those of OTII cells. The effect of Th17 inducing conditions on IL-17 producing cells in TCR-HA transgenic mice was also more comparable with the results obtained using DO11.10 cells. Interestingly, the blocking effect of Th17 polarizing cytokines on Th1 and Th2 cells was less efficient in TCR-HA cells than in DO11.10 and OTII cells.

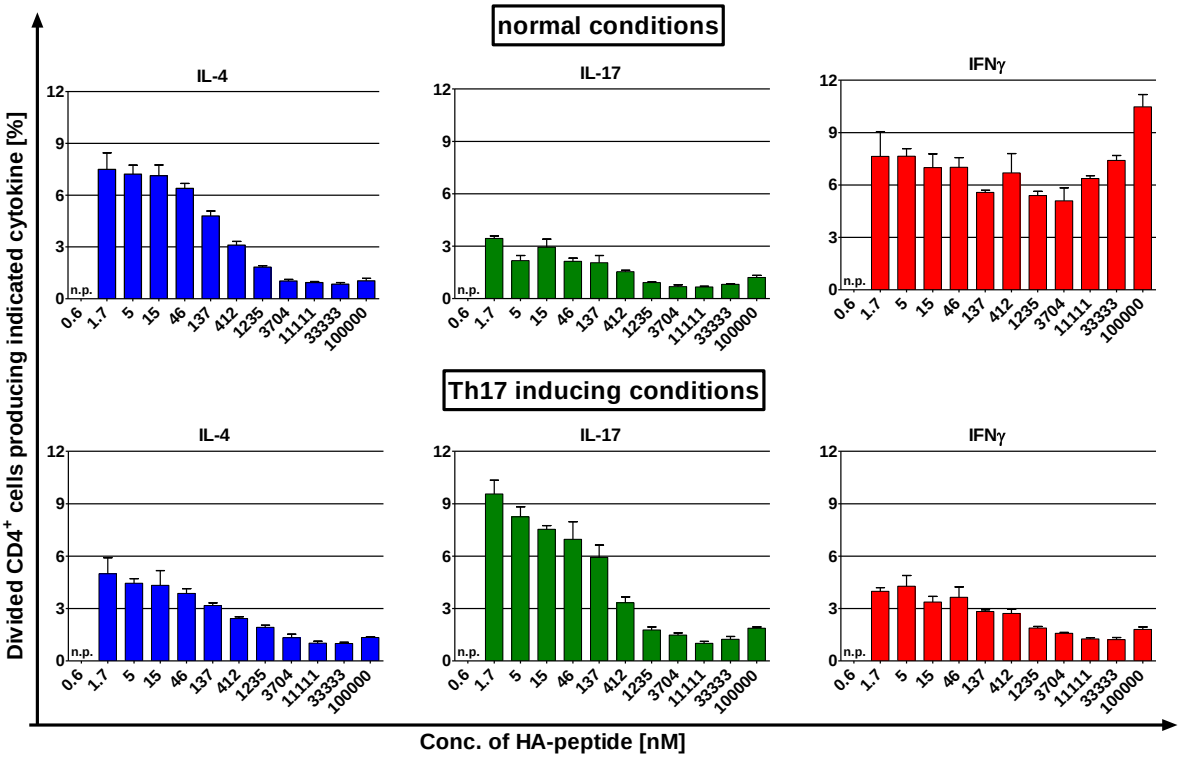

Fig. 30: Cytokine profiles of TCR-HA cells under normal and Th17 polarizing conditions Shown are the Th cytokine profiles of viable and divided $CD4^+$ TCR-HA cells from *in vitro* proliferation assays with different HA-peptide doses. Cells were either cultured under normal conditions or under Th17 inducing conditions. Each bar represents the mean of three samples plus SEM. n.p.= no proliferation.

6.1.4 Studies with FITC-labeled OVA-peptides

One possible explanation for the observed differences in Ag dose dependent Th17 polarization is related to differences in the MHC class II haplotype on DCs obtained from BALB/c mice expressing MHC $H2^d$ or C57BL/6 mice expressing MHC $H2^b$. In fact, the two MHC haplotypes could differ in their affinity to the used peptides, leading to differences in the loading of MHC class II molecules. This can in turn result in different numbers and densities of presented epitopes on DCs, thereby influencing the Ag concentration made available for T cells and subsequently leading to different Th polarizations. To measure the amount of presented OVA-peptide on DCs from either BALB/c or C57BL/6 mice, the peptide was labeled with a fluorochrome. To this end, the OVA-peptide AA323-339 was synthesized with a so-called "spacer", a sequence of seven amino acids at the N-terminus that sticks out of the MHC class II binding grove for peptides. The Serine in the spacer was synthesized as D-isomer to prevent cleavage by proteases. To the N-terminus of the spacer a molecule of the fluorescent organic compound fluorescein (FITC) was linked. For more detailed information and the complete structure see section 4.4. To check if the modification of the peptide is affecting the biological activity, the induction of proliferation in DO11.10 and OTII cells was compared to the unlabeled peptide. For the *in vitro* proliferation assay a peptide concentration inducing maximal T cell proliferation was used (500 nM). Both peptides induced comparable proliferation in DO11.10 and OTII cells, thereby proving that the labeling did not influence the biological activity of the peptide (Fig. 31).

To investigate whether the binding affinity to the OVA-peptide is different, peptide loading experiments with BMDCs derived from BALB/c and C57BL/6 mice were performed. For each peptide concentration, $5x10^5$ sorted BMDCs from each mouse strains were loaded for 3 h and subsequently washed 3 times to remove the peptide that had not been taken up from the samples.

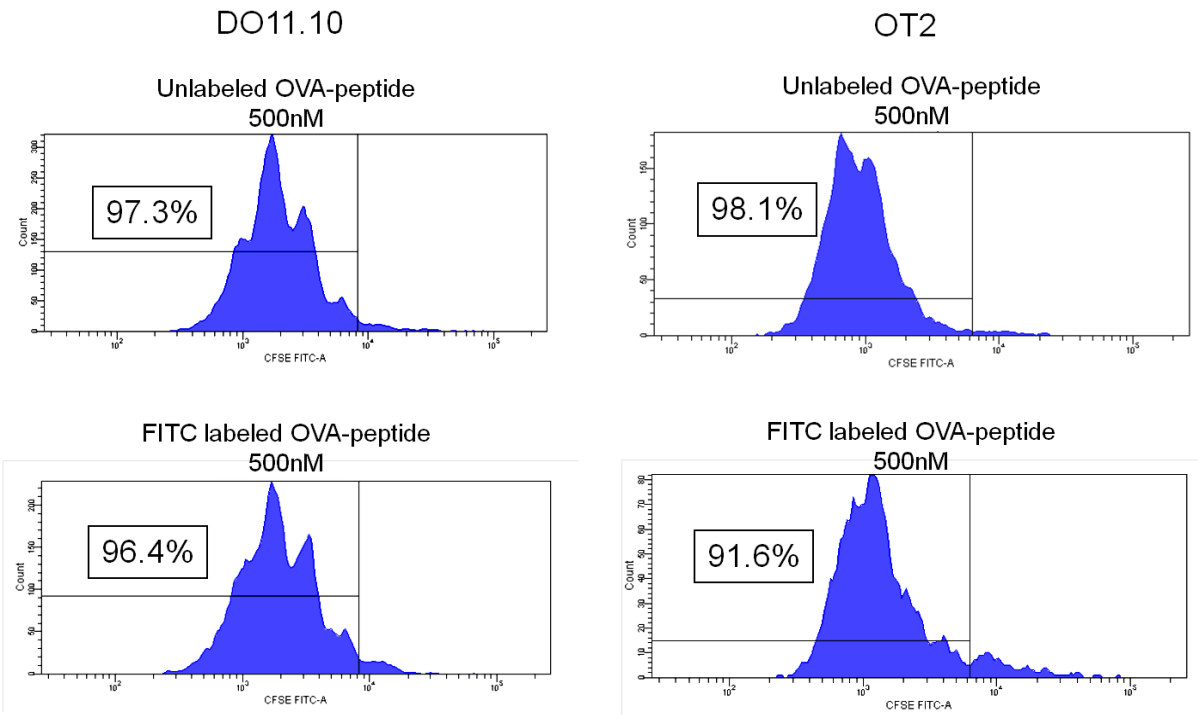

Fig. 31: Biological activity of FITC-labeled OVA-peptide. The FITC-labeled OVA-peptide AA323-339 was compared to the unlabeled peptide at a concentration of 500 nM. The proliferation of sorted DO11.10 and OTII naïve $CD4^+$ T cells was accessed by measuring the dilution of the CFSE signal. Cells were gated on viability and $CD4^+$. Each panel shows the percentage of proliferated cells in a representative out of three independent samples.

The obtained results revealed a high percentage of FITC positive $CD11c^+$ $MHCII^+$ cells for peptide concentrations starting from 412 nM up to maximal Ag dose in both cell types (Fig. 32). At lower peptide concentrations differences in the percentage of loaded DCs were observed in two independent experiments. In the presented experiment peptide loaded DCs derived from C57BL/6 mice (MHC $H2^b$) were detected in higher frequencies than peptide loaded DCs derived from BALB/c mice (MHC $H2^d$). In the second experiment a higher percentage of loading at low Ag doses was observed in DCs derived from BALB/c mice (data not shown). A possible reason for the observed differences might be the limited number of samples. Due to the huge amount of needed DCs, only one sample per Ag concentration for each mouse strain was prepared. However, in another experiment with less Ag doses but three replicates per condition no differences between the DCs of the two mouse strains were observed at low peptide concentrations (see left panel of Fig. 33). This suggests that the affinities MHC $H2^d$ and MHC $H2^b$ molecules to OVA-peptide AA323-339 do not differ. These results rule out that differences in affinity could be responsible for the observed differences in Th17 polarization between DO11.10 and OTII cells.

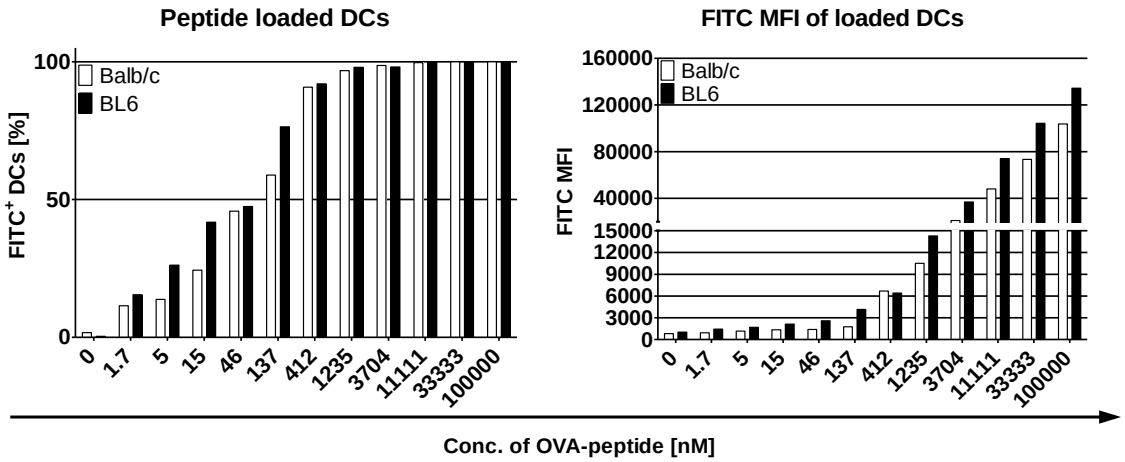

Fig. 32: Loading of FITC-labeled OVA-peptide on BMDCs from BALB/c and C57BL/6 mice. The left panel shows the percentage of CD11c+ MHCII+ cells positive for the FITC-labeled peptide. The right panel shows the median fluorescent intensity (MFI) of FITC from loaded DCs.

6.1.5 Studies with mutated OVA-peptides

In parallel to the previously described experiments with FITC-labeled OVA-peptide AA323-339, peptides with a changed amino acid sequence were synthesized and also labeled with FITC linked to a spacer. The mutated peptides were used to investigate the possibility that differences in TCR affinity to the Ag might be responsible for the observed variations in Th17 polarization. All $CD4^+$ T cells within one TCR transgenic mouse strain display exactly the same TCR with invariant affinity to the specific peptide. Thus, differences in TCRs in DO11.10 and OTII animals may influence the affinity to the OVA-peptide AA323-339. X-ray crystallography experiments revealed the anchor positions of MHC class II and TCR binding to the OVA-peptide AA323-339 [215]. These amino acids are crucial for the peptide binding and an exchange against different amino acids prevents proliferation of DO11.10 and OTII cells. In a study by Sette *et al.* [218] every single amino acid of OVA-peptide AA323-339 was exchanged one by one with alanine and the resulting proliferation of DO11.10 and OTII cells was measured. The exchange at position 335 led to decreased proliferation of DO11.10 cells, whereas OTII cells kept the same proliferation pattern. The opposite effect was observed when exchanging amino acid 336 with alanine. These two mutated OVA-peptides, called in the following “335A” and “336A”, were used for further experiments in this work. Since the structural effect resulting from exchanging asparagine in position 335 or glutamic acid in position 336 with alanine is quite drastic, two additional mutated peptides were generated in which

less severe structural changes were expected. Namely, the peptide “335Q”, in which asparagine was exchanged with glutamine, and the peptide “336D”, where glutamic acid was exchanged with aspartic acid, were designed.

The loading efficiency of these altered peptides at different concentrations on BMDCs derived from BALB/c and C57BL/6 mice was then analyzed. No significant differences in the loading with any of the used peptides were observed independently of the mouse strain (Fig. 33). However, a trend of less efficient loading of BALB/c cells with “335Q” and “336A” was detected. As compared to the normal wild type peptide, especially peptides “335A” and “336D” were loaded less frequently to the DCs at low and medium concentrations.

In vitro proliferation assays with the mutated peptides revealed their strongly impaired biological activities. AA335 mutated peptides showed a 1000 fold decreased capacity to induce proliferation of DO11.10 cells, whereas AA336 altered peptides completely failed to induce robust proliferation of OTII cells at all tested concentrations (data not shown). Due to the impaired proliferation a Th differentiation was also not achieved, and it was thus not analyzed.

The performed experiments did not answer the question if differences in TCR affinity to the OVA-peptide are responsible for the observed differences in Th polarization. However, these results highlighted the importance of TCR affinity, since the influence of altered peptide sequences on T cell proliferation was clearly demonstrated.

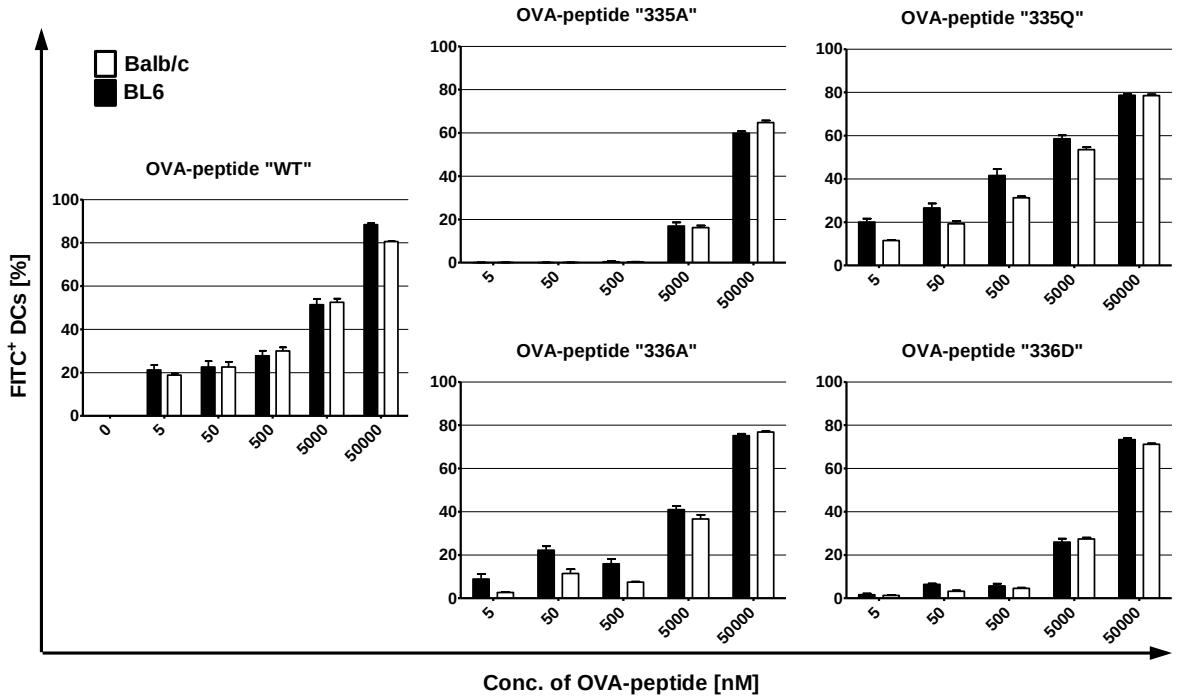

Fig. 33: Loading of FITC-labeled sequence mutated OVA-peptides on BMDCs. The percentage of CD11c+ MHCII+ cells from BALB/c or C57BL/6 mice positive for the indicated FITC-labeled peptide is shown. Each bar represents the mean of three samples plus SEM.

6.1.6 *In vitro* proliferation assay with αCD3 antibody

Another approach to study Th polarization is the stimulation of T cells with αCD3 and αCD28 Abs, which is independent of the TCR affinity to the Ag. The αCD3 Ab binds to the CD3 molecules of the TCR complex and triggers the activation signal cascade. The secondary signal necessary for full T cell activation is provided by αCD28 Ab binding to CD28 on the T cell. Since this method is not dependent on the specificity of TCR to Ag, it is possible to stimulate T cells from wild type mice and thus to study stimulation strength dependent Th polarization. To provide the necessary cell to cell contact to the T cells in this system, BMDCs were added to the cultures at a ratio of 1 to 10. Analogous to the peptide based stimulation described before, the αCD3 Ab concentration inducing T cell proliferation had to be defined. Proliferation started at a αCD3 Ab concentration of 0.06 µg/ml, and reached a maximum at 1-2 µg/ml (data not shown). For the following *in vitro* proliferation assays 1:2 serial dilutions of αCD3 Ab were performed, ranging from 0.06 to 32 µg/ml. The highest proliferation rates were achieved by the addition of 2 µg/ml of αCD28 Ab and were then applied throughout all experiments.

Sorted naïve CD4$^+$ T cells from BALB/c mice were polarized towards a Th2 phenotype at medium and especially at high αCD3 Ab concentrations under normal culture conditions (Fig. 34). Th17 inducing conditions almost completely blocked the induction of IL-4 producing cells. IL-17 producing cells were induced at the lowest Ab concentrations and the overall percentage of IL-17 positive cells was increased under Th17 polarizing conditions. For Th1 cells no real polarization peak was observed, IFNγ producing cells were present in all samples with comparable abundances. Also under Th17 inducing conditions Th1 cells were detected in all samples, but only in 50% decreased levels as compared to the normal culture conditions. The induced Th17 pattern resembles the Ag dose dependent Th17 polarization observed in cells obtained from TCR-HA mice.

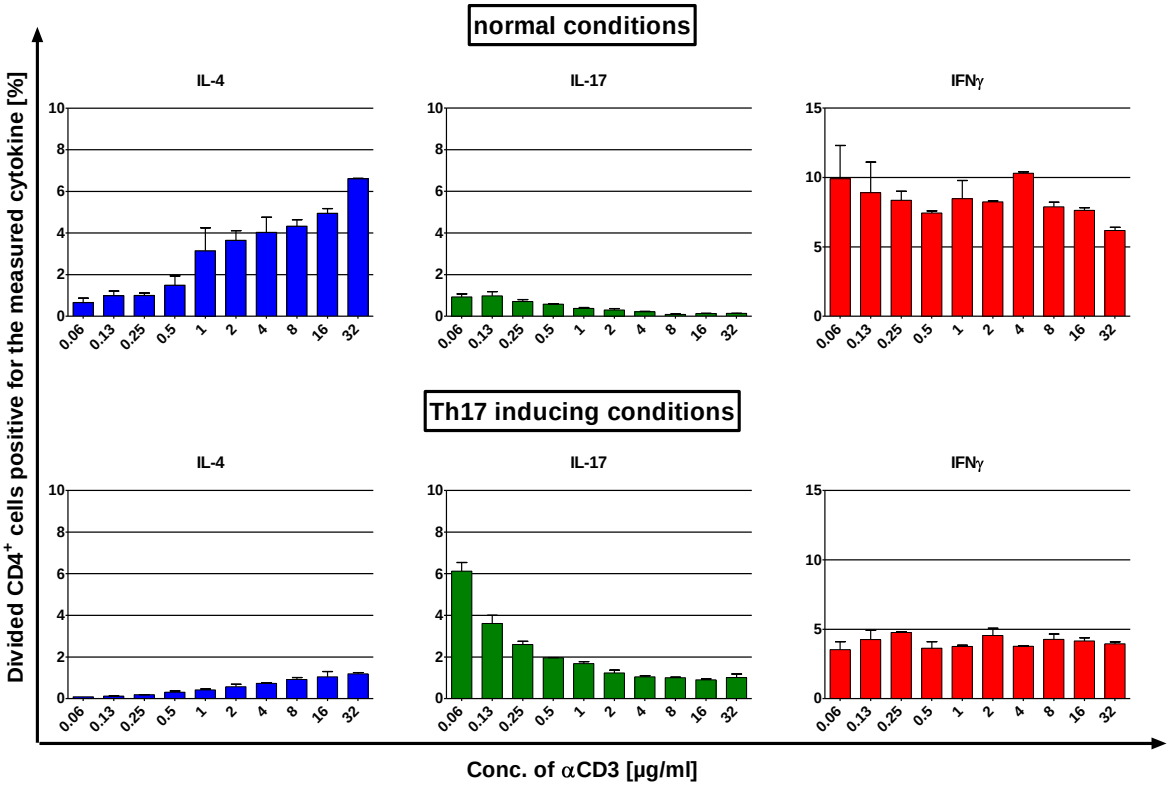

Fig. 34: Cytokine profiles of BALB/c cells under normal and Th17 polarizing conditions following Ag-independent stimulation The Th cytokine profiles of viable and divided CD4$^+$ BALB/c cells from *in vitro* proliferation assays with different αCD3 Ab concentrations are shown. Cells were either cultured under normal conditions or under Th17 inducing conditions in the presence of 2 µg/ml αCD28 Ab. Each bar represents the mean of three samples plus SEM.

Cells from C57BL/6 mice differentiated into a Th2 phenotype at very low and very high stimulus under normal culture conditions (Fig. 35). Th17 inducing conditions blocked the Th2 induction and only at the highest αCD3 Ab concentrations IL-4

producing cells above 0.5% were detected. As already observed for proliferation assays with BALB/c cells, the induction of Th17 cells occurred at low αCD3 Ab doses and was strongly increased under Th17 inducing conditions. IFNγ producing cells were induced at high concentrations and, surprisingly, this induction was increased under Th17 polarizing conditions. Similar to the results obtained with BALB/c cells, the induced Th17 pattern resembles the Ag dose dependent Th17 polarization of TCR-HA cells.

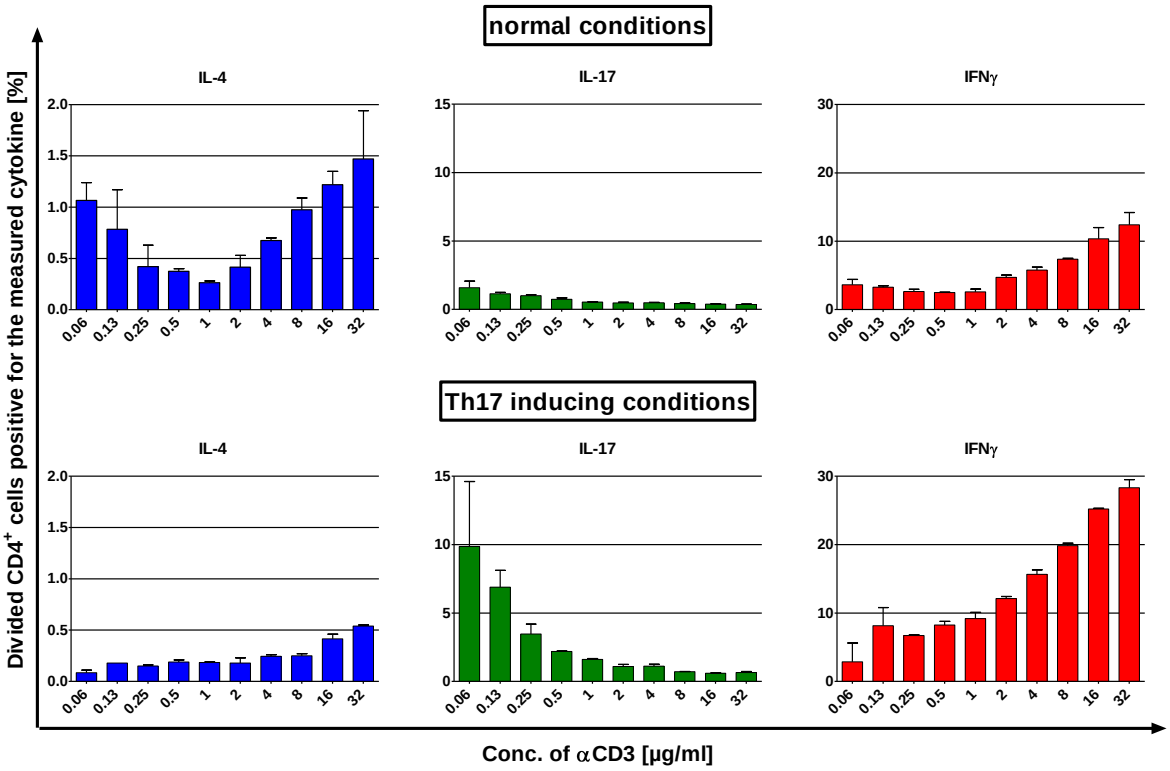

Fig. 35: Cytokine profiles of C57BL/6 cells under normal and Th17 polarizing conditions following Ag-independent stimulation The Th cytokine profiles of viable and divided CD4$^+$ C57BL/6 cells from *in vitro* proliferation assays with different αCD3 Ab concentrations are shown. Cells were either cultured under normal conditions or under Th17 inducing conditions in the presence of 2 µg/ml αCD28 Ab. Each bar represents the mean of three samples plus SEM.

As a next step, Ag-independent Th polarization of cells obtained from TCR transgenic and wild type animals was compared. To this end, sorted naïve CD4$^+$ T cells from OTII animals were stimulated with αCD3 and αCD28 in the presence of C57BL/6 BMDCs. Only weak polarization towards Th2 phenotype was observed under normal culture conditions with two maximums at lowest and at high dose of αCD3 Ab (Fig. 36). Th17 cells were detected especially at low doses but the percentage of Th cells was quite low as compared to *in vitro* proliferation assays

performed with OVA-peptide. The induction of Th1 cells was observed at high stimulus concentrations. An unexpected result was the strong increase of Th1 and Th2 induction under Th17 polarizing conditions. Th1 cells peaked at very high Ab doses, whereas Th2 cells had a maximum at 4 µg/ml of αCD3 Ab. Also the polarization to IL-17 producing cells was increased. At a concentration of 0.06 µg/ml of αCD3 Ab, the percentage of Th17 cells raised from 1% to 15%.

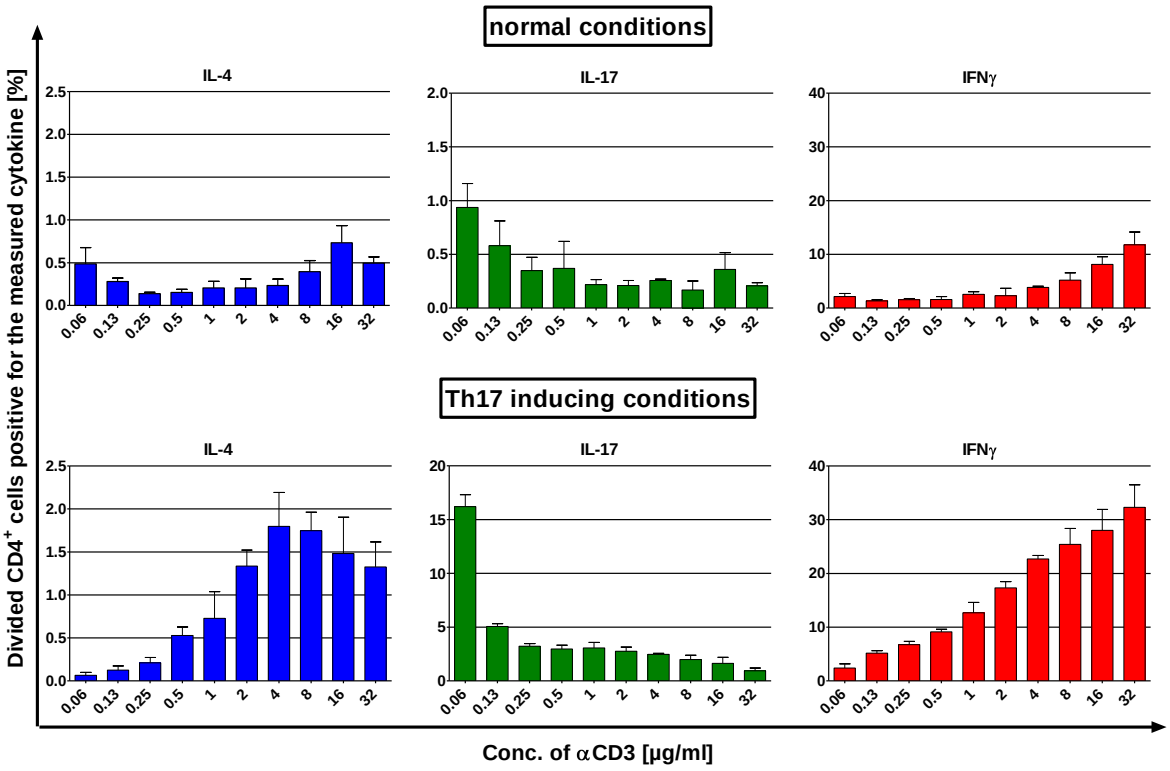

Fig. 36: Cytokine profiles of OTII cells under normal and Th17 polarizing conditions following Ag-independent stimulation The Th cytokine profiles of viable and divided $CD4^+$ OTII cells from *in vitro* proliferation assays with different αCD3 Ab concentrations are shown. Cells were either cultured under normal conditions or under Th17 inducing conditions in the presence of 2 µg/ml αCD28 Ab. Each bar represents the mean of three samples plus SEM.

These experiments clearly demonstrated an induction of Th17 cells by low strength stimulation in wild type mice as well as in TCR transgenic OTII animals. Thus, it can be hypothethized that the observed absence of Th17 polarization when OTII cells were stimulated with low doses of the OVA-peptide AA323-339 can be explained, at least in part, by differences in TCR affinity.

6.1.7 Adoptive transfer of TCR transgenic T cells to wild type recipient mice

For studying Ag dose dependent Th polarization of OTII cells in a more natural setting, adoptive transfer experiments were performed. To identify the transferred TCR transgenic cells in the wild type C57BL/6 host, Thy1.1xOTII mice were used as donors. Thy1.1 (or CD90.1) is a so-called congenic marker. Wild type mouse strains express the “normal” Thy1.2 molecule, which differs slightly from Thy1.1 in structure but not in function. Non cross-reactive monoclonal Abs against each form of Thy1 are available and can be used to distinguish transferred from host cells. Naïve $CD4^+$ T cells from Thy1.1xOTII double transgenic animals were sorted by FACS and labeled with CFSE. Cells were injected in the tail vein of each C57BL/6 recipient mouse. The next day, mice were immunized with 25µg OVA and 100µg Curdlan intra footpad. The draining lymph node of the foot pad is the popliteal lymph node (pLN) which is located at the hollow of the knee. To define the ideal time point after injection to measure proliferation and Th differentiation of transferred cells, three different time points were tested (1, 2 and 3 days post immunization). The pLNs from animals collected on day two and three were clearly larger than the pLNs from negative control mice and mice analyzed 1 day post immunization. Single cell suspensions from pLNs and spleens were stained with FACS Abs for viability, CD4, Thy1.1, IL-4, IL-17 and IFNγ.

The recovered fractions of transferred cells in the host animals were quite constant between day 1 and 2 post immunization in pLNs and spleen (Fig. 37). A huge increase of transferred cells was observed in pLNs as well as in the spleens after 3 days. The proliferation of transferred cells was measured by CFSE dilution and is shown as histograms in Fig. 37. After one day no divided cells were found in pLNs or spleen. Proliferation started at day 2 in the draining LNs and one day later almost all transferred cells detected in pLNs and spleen were activated and divided. To determine the induced Th phenotype of the divided cells, their cytokine profiles were analyzed. Cells from pLNs on day 2 did not produce cytokines (data not shown) and on day 3 cytokines were only detected in the cells isolated from pLNs (Fig. 38). Mainly Th17 and Th2 cells were induced by immunization, whereas IFNγ positive cells were almost not detectable.

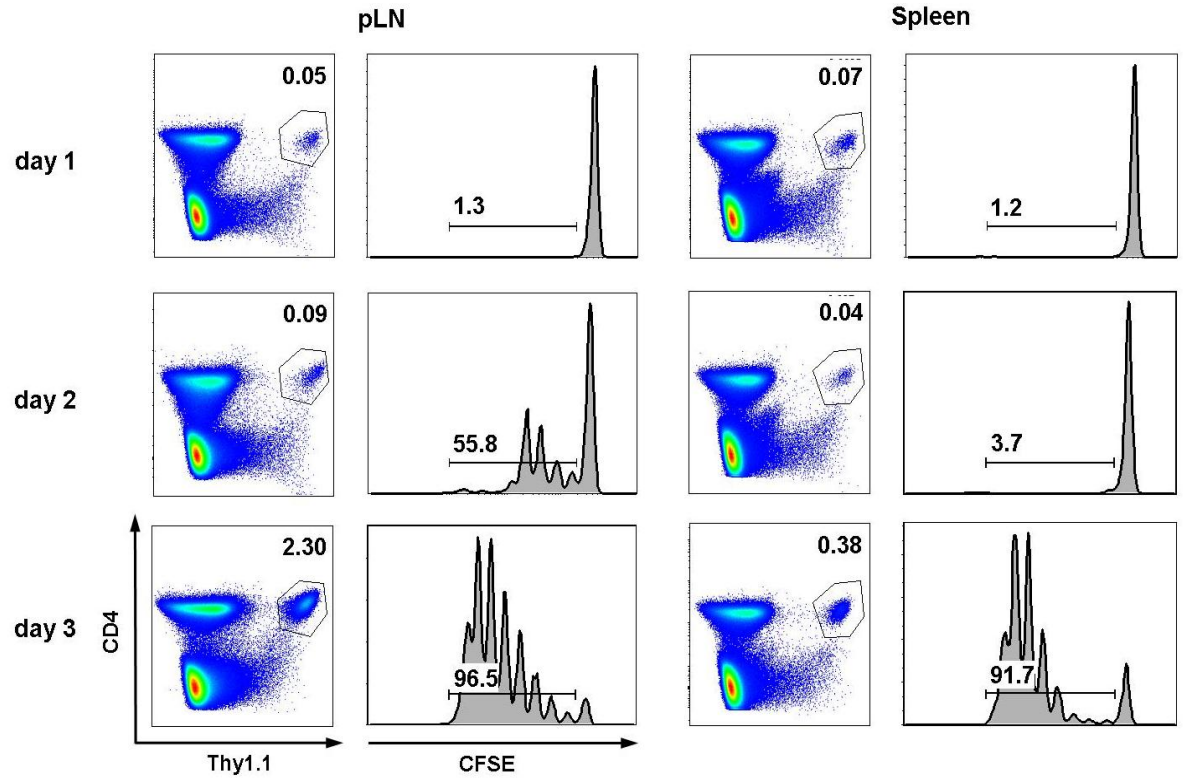

Fig. 37: Time kinetics of T cell proliferation after adoptive transfer. The dot plots show the population of adoptively transferred Thy1.1xOTII cells in pLNs or spleen at indicated time points after intra footpad immunization with 25 µg OVA and 100 µg Curdlan. Histograms show the proliferation of transferred cells, as measured by CFSE dilution. Data for each day are from one mouse representative for the group.

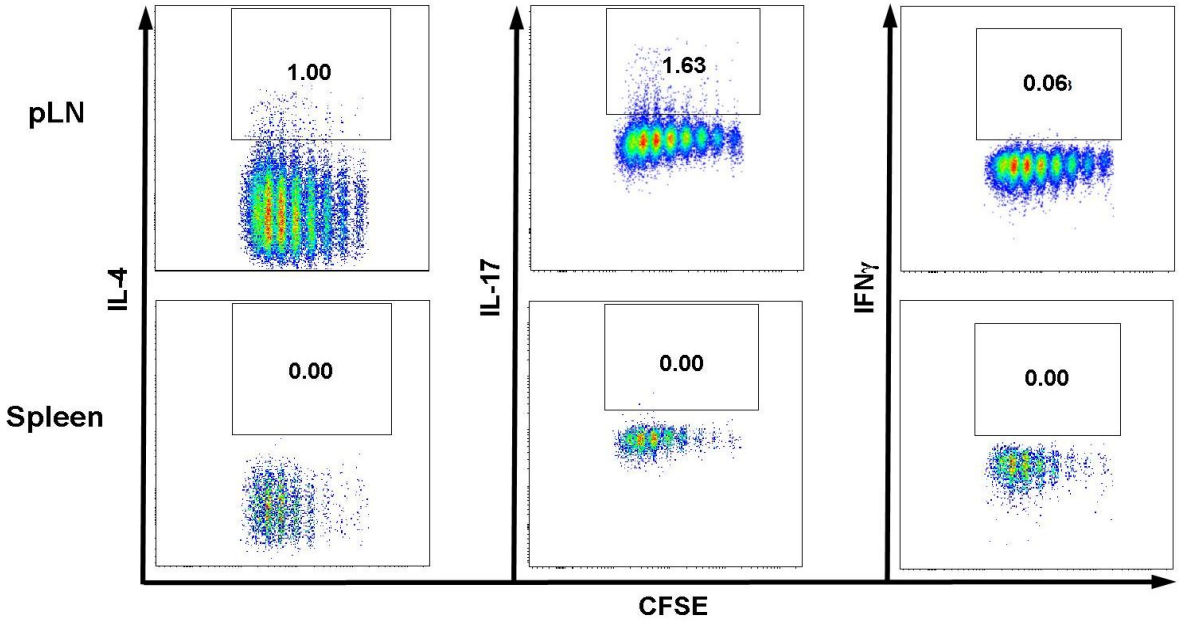

Fig. 38: Cytokine profiles of adoptively transferred cells. Shown is the production of IL-4, IL-17 and IFNγ by adoptively transferred Thy1.1xOTII cells isolated from pLNs or spleen 3 days post intra footpad immunization with 25 µg OVA and 100 µg Curdlan.

Following these results, nine groups of three C57BL/6 mice were immunized for 3 days with different concentrations of OVA co-administered with Curdlan by intra footpad injection after adoptive transfer of CFSE-labeled naïve $CD4^+$ Thy1.1xOTII cells. Negative control animals received PBS. To compare the groups, the percentage of transferred cells isolated from recipient pLNs were analyzed and investigated with respect to their proliferative capacity (Fig. 39). In the PBS group, the adjuvant control group, and the two groups with either lowest or highest Ag dose in combination with Curdlan only small populations (<0.3%) of transferred cells were detected. In the OVA control group and all intermediate Ag dose groups comparable populations of transferred cells were observed. However, between the single animals in some cases big differences in the percentage of transferred cells were observed; especially in the 1 µg OVA and Curdlan group. The proliferation rates of transferred cells rose with the applied Ag dose and reached a maximum beginning with 25 µg of OVA (almost 100%). An influence of the adjuvant on proliferation was not observed, since also the cells of the control group immunized with OVA alone showed full proliferation.

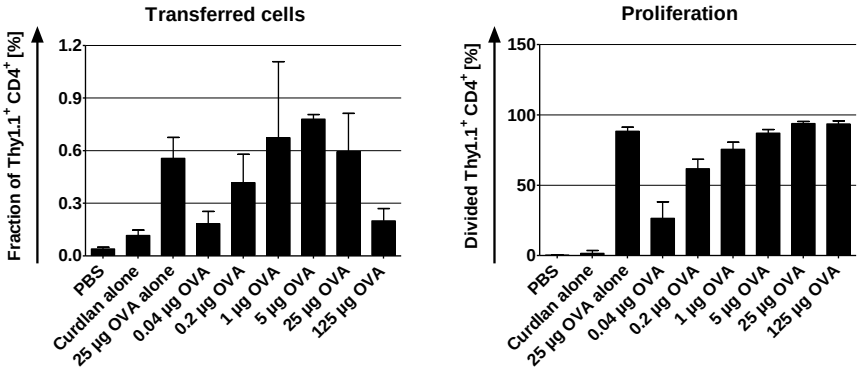

Fig. 39: Characterization of adoptively transferred Thy1.1xOTII cells. The left panel shows the percentage of $Thy1.1^+$ $CD4^+$ T cells in pLNs from animals which were immunized with different doses of OVA + Curdlan after adoptive transfer of 2.5 x 10^6 naïve $CD4^+$ Thy1.1xOTII cells 4 days before. Right panel shows the proliferation of these cells measured by CFSE dilution. Every animal of each group (n = 3) was analyzed separately and the mean plus SD is shown.

Th cytokine profiles of proliferated transferred cells were analyzed utilizing the established cytokine FACS panel. In the PBS and Curdlan control group no cytokine producing cells were detected, whereas some positive cells for each cytokine were found in the OVA control group (Fig. 40). IL-4 production was observed at all Ag concentrations in equal frequencies (around 0.5%). Only in the group immunized with

5 µg OVA and Curdlan higher percentages of IL-4 producing cells were found. The strongest polarization toward Th17 was detected in mice immunized with the lowest Ag dose. Immunization with 25 µg and 125 µg OVA resulted in the induction of intermediate Th17 responses, whereas 5 µg OVA resulted in low frequencies of IL-17 producing cells. The complete opposite picture was observed when looking on Th1 cells. A clear increase of IFNγ producing cells was detected in groups immunized with the two highest OVA doses, as compared to lower Ag doses.

However, the analysis of cytokine profiles showed strong variations between animals, leading to high standard deviations. This explains that the observed differences in IL-17 production were not statistically significant, despite the fact that the observed trend suggests a stronger Th17 polarization at the lowest Ag dose. This trend would contradict the observations from *in vitro* experiments using OVA-peptide for stimulation of OTII cells. However, it would be in agreement with the Ag-independent stimulation of OTII cells by αCD3 Ab, which led to Th17 induction when low Ab concentrations were used. Further, it is possible that *in vivo* and *in vitro* conditions result in different polarization behavior of TCR transgenic cells.

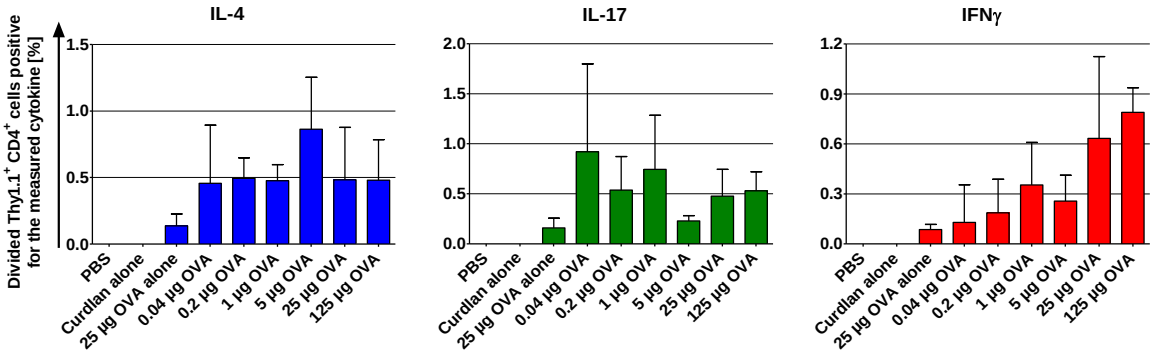

Fig. 40: Cytokine profiles of adoptively transferred Thy1.1xOTII cells. Production of IL-4, IL-17 and IFNγ by viable and divided adoptively transferred $CD4^+$ Thy1.1xOTII cells, isolated from pLNs 3 days post intra footpad immunization with different doses of OVA and 100 µg Curdlan is shown. Every animal of each group was analyzed separately and the mean plus SD is shown.

6.1.8 Immunization studies with different dosages of OVA-protein

The presented *in vitro* studies together with the in vivo passive transfer experiments strongly suggested that under natural conditions a Th17 immune response is elicited by low Ag doses. To validate these observations, the influence of Ag dose on Th polarization *in vivo* was evaluated (Fig. 41). Groups of three or five C57BL/6 animals were immunized by either i.p. or s.c. route with different dosages of OVA-protein and 20 or 25 µg of LPS on day 0 and boosted on day 14 and day 21. At day 42 splenocytes were analyzed with respect to cytokine secreting cells and proliferative capacity by ELISpot and ^{3}H-thymidin incorporation assays. In parallel to the titration of the OVA-protein, also a titration of LPS at constant OVA concentration of 25 µg per animal was performed in the i.p. immunization. A dose of 25 µg of OVA per animal are routinely used to induce a robust immune response against the Ag and will be regarded as “standard dose” in the following.

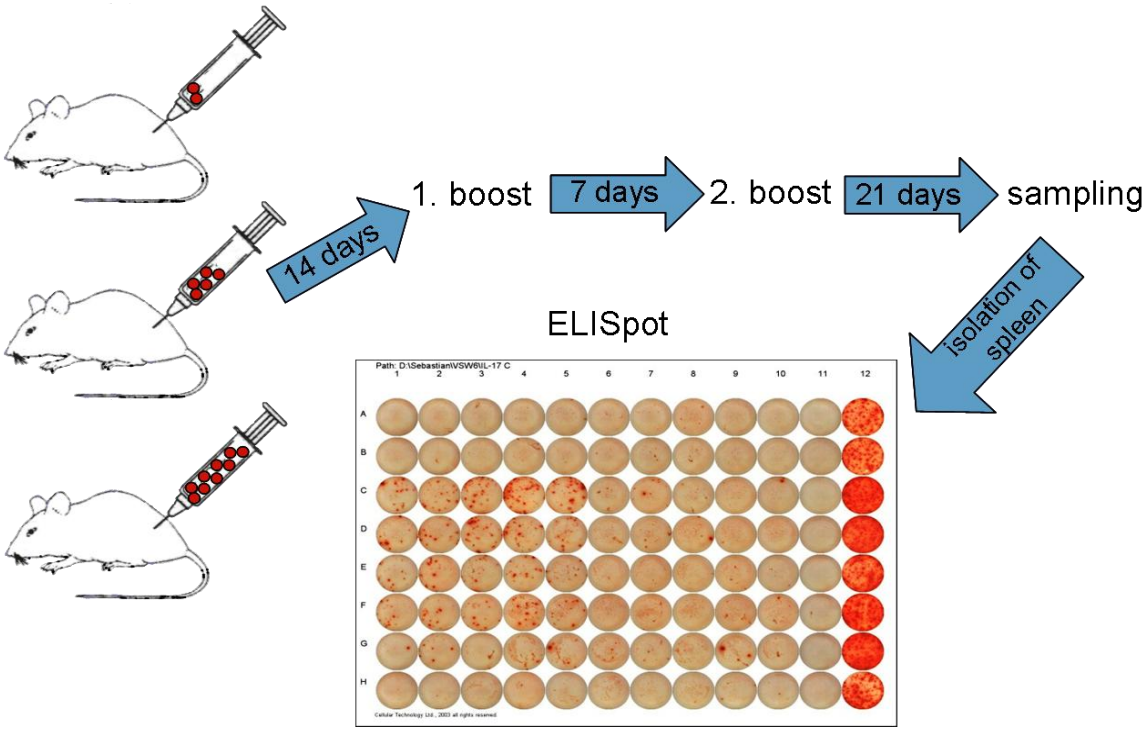

Fig. 41: Schematic illustration of the immunization strategy. Mice were immunized either by i.p or s.c. route with varying doses of OVA+LPS. The animals were boosted with the same OVA+LPS combination on day 14 and 21. After a total of 42 days, mice were sacrificed and spleens were isolated for read out of the immune response, which was measured by performing ELISpot analysis and ^{3}H-Thymidin proliferation assays.

As expected, in the groups immunized with PBS or LPS alone, only few IL-17 secreting cells were detected after restimulation with OVA (Fig. 42). The lowest Ag dose only resulted in a weak Th17 immune response, whereas the immunization with 1 µg of OVA led to significantly increased IL-17 responses as compared to the standard dose or even higher Ag concentrations. Titration of the co-administered LPS dose revealed a decreased number of Th17 cells when lowering the LPS dosage under 25 µg per animal. An increase of the LPS dose instead resulted in a significant higher number of IL-17 producing cells, as compared to standard immunization conditions. The groups immunized with OVA and LPS displayed an increased Ag specific proliferation, as compared to the negative control groups (Fig. 43). Only the combination of 125 µg OVA and 25 µg LPS resulted in low levels of thymidine incorporation.

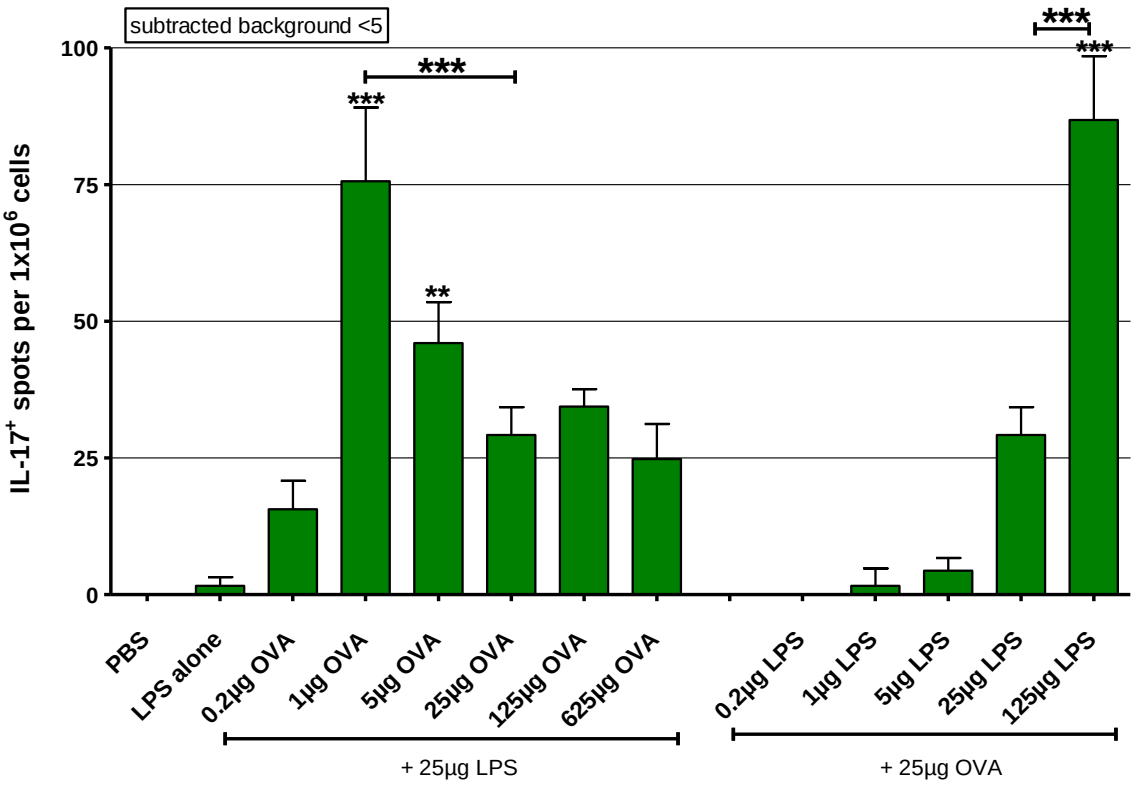

Fig. 42: IL-17 response of splenocytes after i.p. immunization with OVA+LPS. C57BL/6 mice were immunized three times with varying doses OVA+LPS by i.p. route. Spleens were pooled and IL-17 ELISpot assays were performed on OVA restimulated cells. Each bar represents the mean of five wells plus SEM; background was subtracted. **:p<0.01; ***:p<0.001; as compared to LPS alone or between groups as indicated. n=3

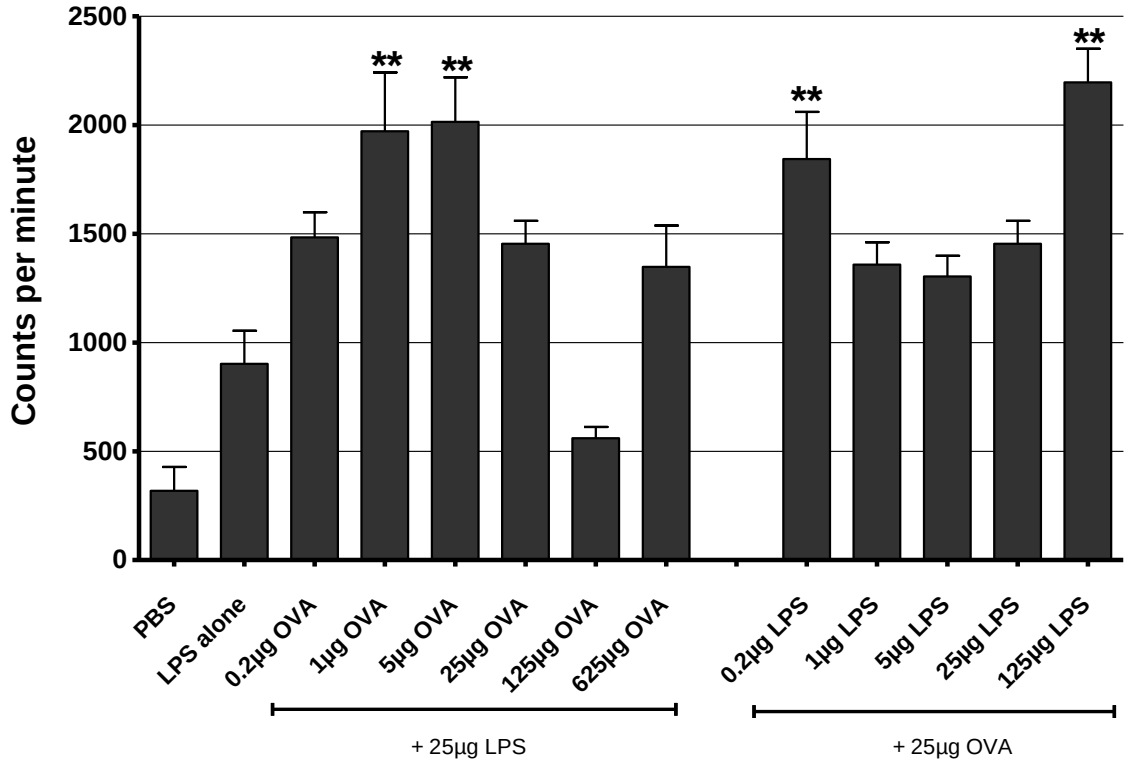

Fig. 43: Proliferation of splenocytes after i.p. immunization with OVA+LPS. C57BL/6 mice were immunized three times with varying doses OVA+LPS i.p.. Spleens were pooled and a ^{3}H-Thymidin incorporation assay was performed on OVA restimulated cells. Each bar represents the mean of four wells plus SEM. **:p<0.01; compared to LPS alone. n=3

To investigate if the observed induction of Th17 cells at low Ag concentrations was dependent on the route of immunization, an experiment with application of OVA and LPS by the s.c. route was performed. An IL-17 ELISpot performed with pooled splenocytes from the immunized mice revealed a significant increase of Th17 cells at the lowest Ag dose, as compared to the standard dose of 25 µg (Fig. 44). Immunization with higher Ag concentrations resulted in decreased Th17 induction. In PBS and LPS control groups no induction of IL-17 producing cells was detected. To evaluate the induction of Ag specific proliferation of immune cells by the immunization, a ^{3}H-Thymidin incorporation assay was also performed. As expected, in cells from the two negative control groups very weak proliferation was observed as compared to groups immunized with Ag and LPS (Fig. 45). High Ag doses resulted in quite poor induction of specific immune cells, whereas the proliferation rate at the two lowest doses was modest, as compared to other groups. Strong proliferation was measured at the intermediate Ag concentrations of 5 and 25 µg per animal. The differences in optimal dosage for Th17 cells induction can be attributed to the fact

that s.c. vaccination is more efficient, since a higher number of APCs is locally available.

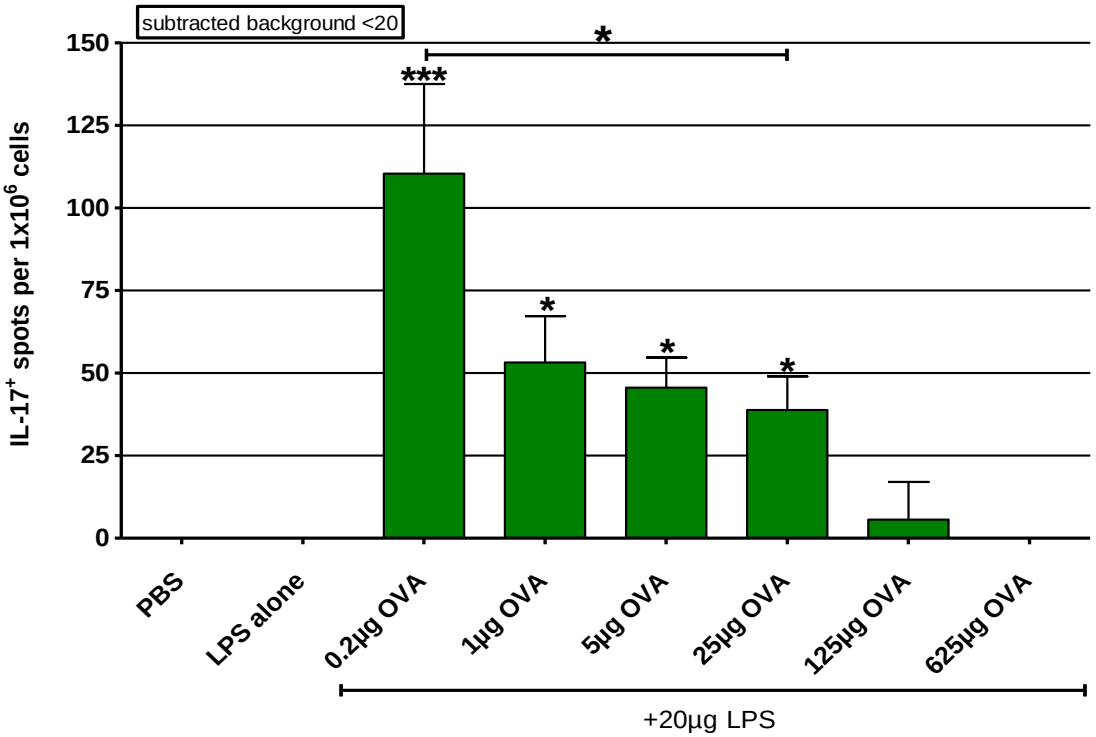

Fig. 44: IL-17 response of splenocytes after s.c. immunization with OVA+LPS. C57BL/6 mice were immunized three times with varying doses OVA+LPS by s.c. route. Spleens were pooled and IL-17 ELISpot assay was performed on OVA restimulated cells. Each bar represents the mean of five wells plus SEM; background was subtracted. *:p<0.05; ***:p<0.001; compared to LPS alone or between groups as indicated. n=5

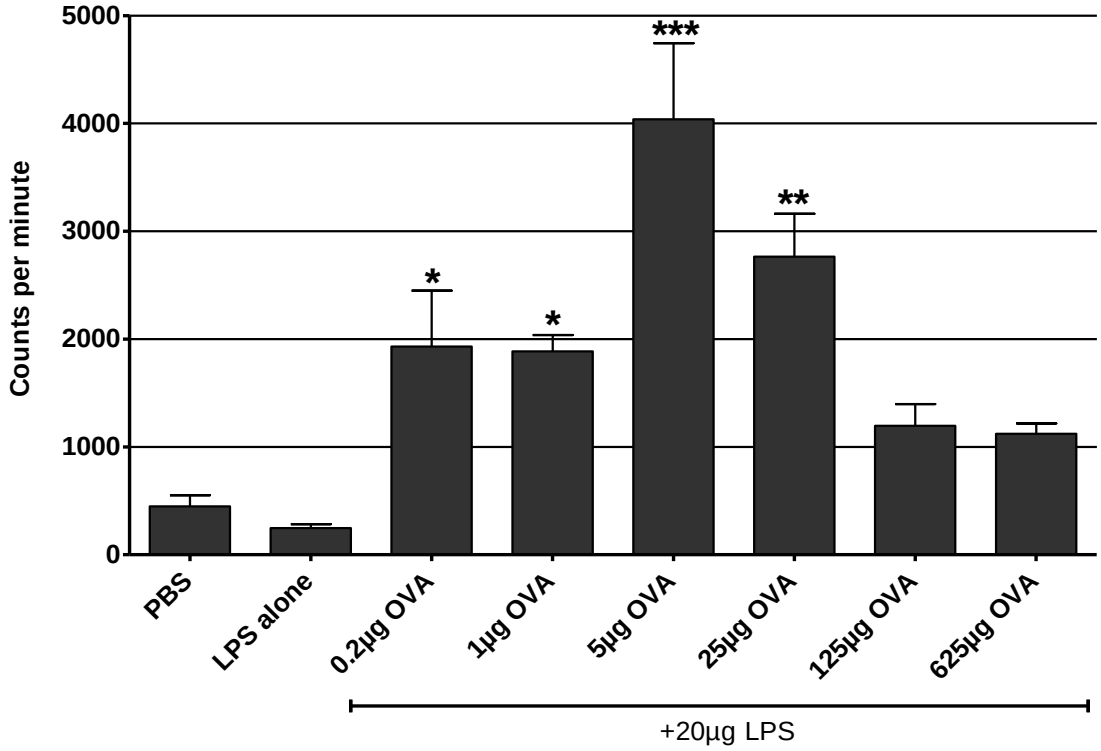

Fig. 45: Proliferation of splenocytes after s.c. immunization with OVA+LPS. C57BL/6 mice were immunized 3 times with varying doses OVA+LPS by s.c. route. Spleens were pooled and ^{3}H-Thymidin incorporation assay was performed on OVA restimulated cells. Each bar represents the mean of 4 wells plus SEM. *:p<0.05; **:p<0.01; ***:p<0.001; compared to LPS alone. n=5

Taking the proliferation and ELISpot data under account, a specific Th17 response was observed at low Ag concentrations, regardless of the route of application. These results gave further evidence that Th17 immune responses are normally elicited under conditions of weak Ag stimulation, as already observed *in vitro* when stimulating $CD4^{+}$ T cells with αCD3 Ab.

6.2 Effect on Th polarization and protective efficacy against the influenza virus resulting from vaccination using different Ag doses

The above presented immunization studies with the model Ag OVA revealed that Th17 induction can be promoted in mice at low protein concentrations. Next, it was investigated if *in vivo* Th17 polarization at low antigen dosages is a general phenomenon or only related to certain Ags like OVA. It was decided to use a vaccine relevant Ag, which would also allow to address the potential impact of Th17 polarization in overall protective efficacy conferred by the vaccine. To this end, mice were immunized with different concentrations of virosomes generated using Ags from

the avian influenza virus H5N1. These virosomes are small phospholipid bilayer particles, which present HA5 and NA1 molecules on their surface. They resemble the influenza virus and can enter host cells via HA mediated phagocytosis. As a consequence, immune responses against HA and NA are elicited. However, since virosomes contain no genetic material, they cannot replicate (see also 2.3).

BALB/c mice were immunized on day 0, 14 and 21 by i.n. route with different dosages of H5N1 virosomes co-administered with c-di-AMP as adjuvant. Parts of the animals were analyzed 42 days after priming with respect to their Ag specific immune responses in terms of proliferation and the induced Th phenotypes. The remaining mice were challenged by i.n. application of a lethal dose of H5N1 virus (strain NIBRG-14) and their survival was monitored for the following 6 days.

Immunization with 0.5, 2.5 and 7.5 µg (HA equivalents) of H5N1 virosomes plus c-di-AMP led to strong Ag specific proliferation of spleen cells, as compared to the negative control groups and the group which received the lowest Ag concentration (Fig. 46). Strongest proliferation was observed in the group immunized with 2.5 µg virosomes plus adjuvant when cells were restimulated with 2 µg/ml of virosomes. With cells from the groups receiving 0.5 and 7.5 µg, the strongest proliferation was detected when cells were restimulated with 0.1 and 1 µg of Ag, respectively. These results demonstrate that already 0.5 µg of H5N1 virosomes in combination with c-di-AMP are able to elicit a robust Ag specific cell pool after immunization.

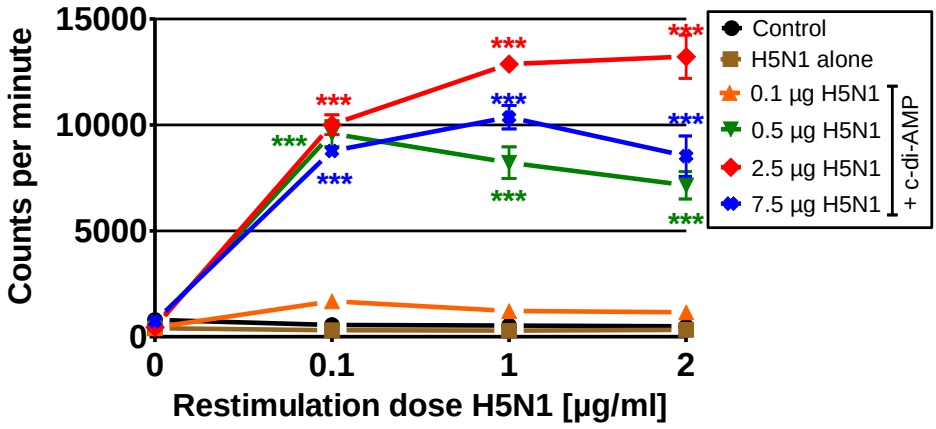

Fig. 46: Proliferation of splenocytes after i.n. immunization with H5N1 virosomes and c-di-AMP. BALB/c mice were immunized three times with different dosages of H5N1 virosomes + c-di-AMP by i.n. route. Spleens were pooled and a ^{3}H-Thymidin incorporation assay was performed after *in vitro* restimulation with indicated Ag doses. Each point represents the mean of four wells plus SEM. ***: $p < 0.001$; compared to H5N1 alone. n=5

To investigate Th immune responses, cytokine secretion by splenocytes was assessed by ELISpot assay. Between samples from mice immunized with 0.5, 2.5 or 7.5 µg virosomes plus c-di-AMP no significant differences were observed (Fig. 47). They all showed high numbers of IL-17 and IFNγ producing cells and only few cells positive for IL-4. In the 2.5 µg plus adjuvant group a slightly increased number of IFNγ positive cells was detected, as compared to the other groups. The signal of mice immunized with 0.1 µg virosomes and c-di-AMP was very weak. In samples from PBS and Ag alone control groups, no spot formation above background for IL-4, IL-17 and IFNγ was observed. These results suggest a Th1/Th17 dominated immune response elicited by co-administration of at least 0.5 µg H5N1 virosomes and c-di-AMP.

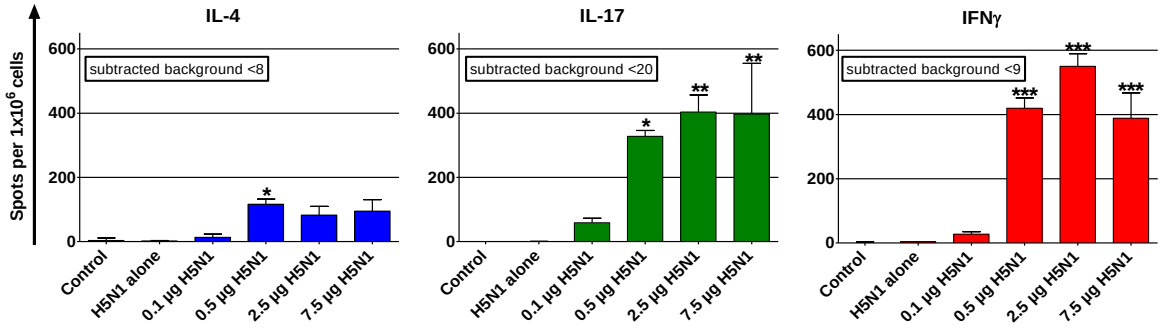

Fig. 47: Cytokine profiles of splenocytes after i.n. immunization with H5N1 virosomes and c-di-AMP. BALB/c mice were immunized three times with different dosages of H5N1 virosomes + c-di-AMP by i.n. route. Spleens were pooled and ELISpot assays for IL-4, IL-17 and IFNγ were performed. Each bar represents the mean of six wells plus SEM. *: p<0.05; **:p<0.01; ***:p<0.001; compared to H5N1 alone. n=5

On the other hand, the production of IL-4, IL-17 and IFNγ is not unique to Th cells. Thus, it had to be confirmed if Th cells were indeed the source of the detected cytokines. This was achieved by the establishment of a multicolor FACS staining panel to characterize Ag specific immune responses from *in vivo* samples. The panel had to fulfill the two following tasks: (i) identification of all Th subsets and (ii) characterization of polyfunctional $CD4^+$ T cells, which have been shown to play an important role in conferring protection against infections. Polyfunctional $CD4^+$ T cells are characterized by their ability to produce at least two out of the three cytokines IL-2, IFNγ and TNFα. To match these requirements it was necessary to set up a staining with nine different markers: Live/Dead, CD3, CD4, CD8, IL-2, IL-4, IL-17, IFNγ and TNFα. This combination of markers offers the possibility to measure also $CD8^+$ T cell cytokine responses. The successful establishment of a staining protocol with working combinations of markers and fluorochromes is a powerful tool for T cell

analysis. This rendered possible the analysis of Ag specific cytokine profiles of restimulated $CD4^+$ T cells from the mice immunized with H5N1 virosomes co-administered with c-di-AMP. Almost no IL-4 producing cells were detected among all groups (Fig. 48). Only in the groups receiving virosomes co-administered with c-di-AMP Ag specific responses were observed, as demonstrated by detection of intracellular IL-2, IL-17 and IFNγ. As compared to IL-2 and IL-17 producing cells (both about 0.3%), only few cells were positive for IFNγ (about 0.1%). On the other hand TNFα production was detected in all samples, but only in the groups which received Ag plus adjuvant the TNFα fractions were increased in Ag restimulated samples. Between cells from different virosome immunization groups, no significant differences with respect to cytokine responses were observed.

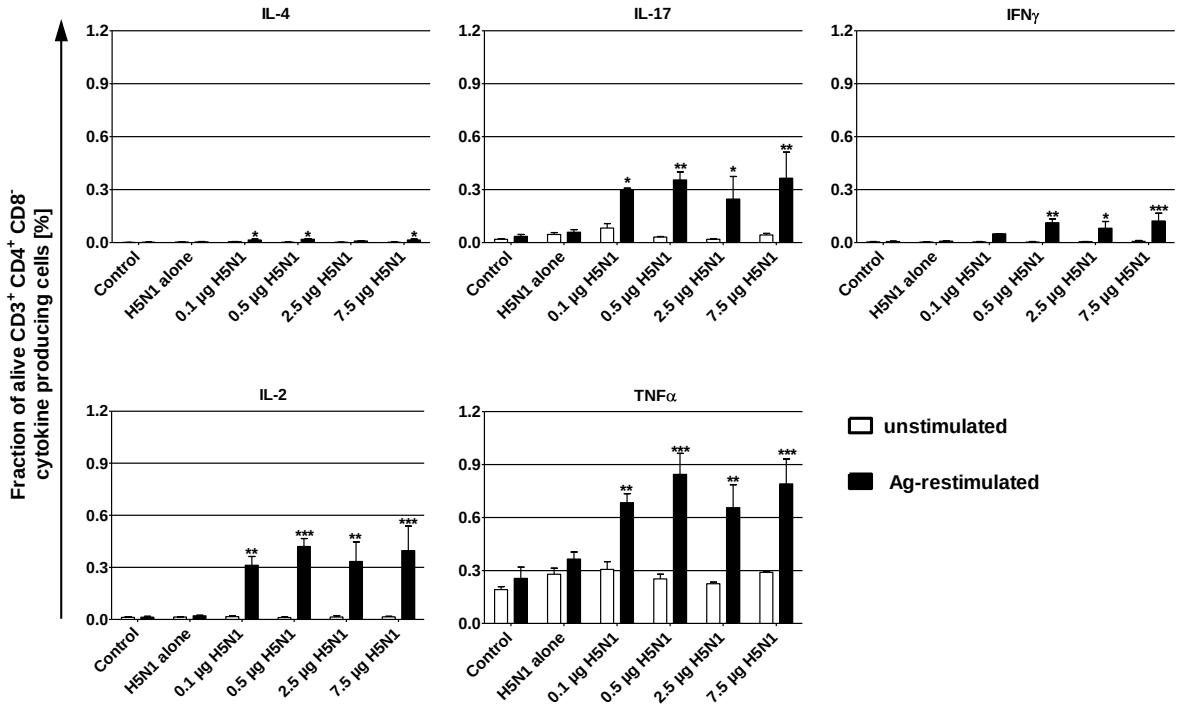

Fig. 48: Cytokine profiles of splenocytes after i.n. immunization with H5N1 virosomes and c-di-AMP. BALB/c mice were immunized three times with different dosages of H5N1 virosomes + c-di-AMP by i.n. route. Spleens from three animals per group were separately restimulated with H5N1 virosomes and stained for viability, CD3, CD4, CD8, IL-2.IL-4, IL-17, IFNγ and TNFα and analyzed by FACS. Each bar represents the mean of three mice plus SD. *: p<0.05; **:p<0.01; ***:p<0.001; unstimulated compared to Ag-restimulated. n=5

To characterize polyfunctional $CD4^+$ T cells, the profiles of cytokine secreting cells were analyzed by Boolean gating to identify cells producing combinations of IL-2, IFNγ and TNFα. In control groups receiving PBS or virosomes alone, almost no double or triple cytokine producing cells were detected (Fig. 49). Cells positive for

two cytokines were found in comparable numbers in all four groups immunized with Ag and adjuvant. The lowest percentages were detected in the 0.1 µg group and highest frequency in the 0.5 µg group. The double positive cells produced mainly IL-2 in combination with TNFα, whereas the two other combinations of co-production were observed only in few cells. The production of all three was only detected at a maximum of 0.03% of viable, $CD3^+$ $CD4^+$ $CD8^-$ cells in mice immunized with the highest virosome dosage. In groups immunized with 0.1 or 2.5 µg of virosomes co-administered with c-di-AMP only 0.01% of triple positive cells were observed, whereas in the 2.5 µg group a mean value of about 0.02% was measured.

This analysis of cytokine profiles demonstrated that the immunization with H5N1 virosomes co-administered with c-di-AMP induces not only normal Th responses, but also polyfunctional $CD4^+$ T cells. However, an Ag dose dependent effect was not observed.

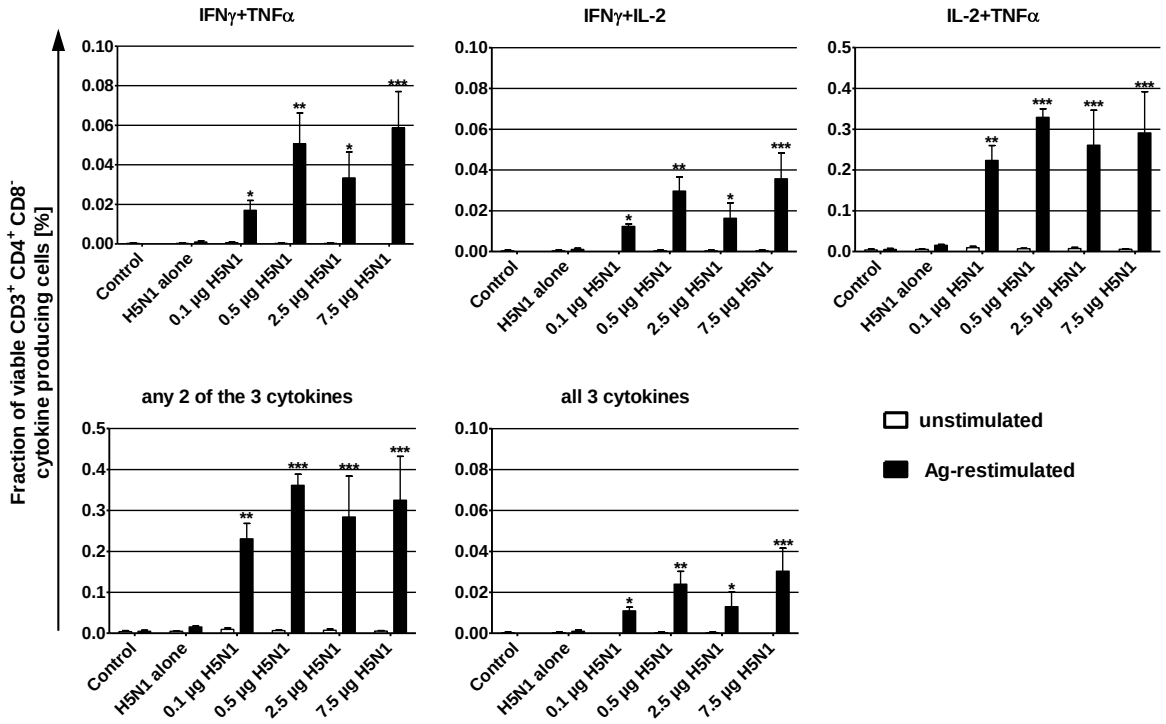

Fig. 49: Analysis of polyfunctional T cells after i.n. immunization with H5N1 virosomes and c-di-AMP. Viable and polyfunctional $CD4^+$ $CD8^-$ T cells were identified by Boolean gating on double or triple producers of IL-2, IFNγ and TNFα shown in Fig. 48. Each bar represents the mean of three mice plus SD. *: p<0.05; **:p<0.01; ***:p<0.001; unstimulated compared to Ag-restimulated.

To check whether immunization with H5N1 virosomes at different doses and co-administration with c-di-AMP as adjuvant is able to protect against infection, the

remaining animals of each group were challenged by i.n. route with the H5N1 influenza virus. On the following 6 days the weight and general health of each mouse was monitored twice a day. Animals were sacrificed if they showed weight reduction of 25%, as compared to the starting weight. Mice immunized with PBS or virosomes alone died within 3 or 4 days post challenge (Fig. 50). Only one mouse of the PBS control group showed no symptoms of infection at all. Animals immunized with 0.5, 2.5 or 7.5 µg of virosomes plus c-di-AMP were fully protected and only mild transient weight loss in the first days was detected (data not shown). In the group which received the lowest Ag dose during immunization, every third animal died or had to be sacrificed. Also the surviving animals of this group showed more severe symptoms of acute influenza infection, such as weight loss and scrubby hair.

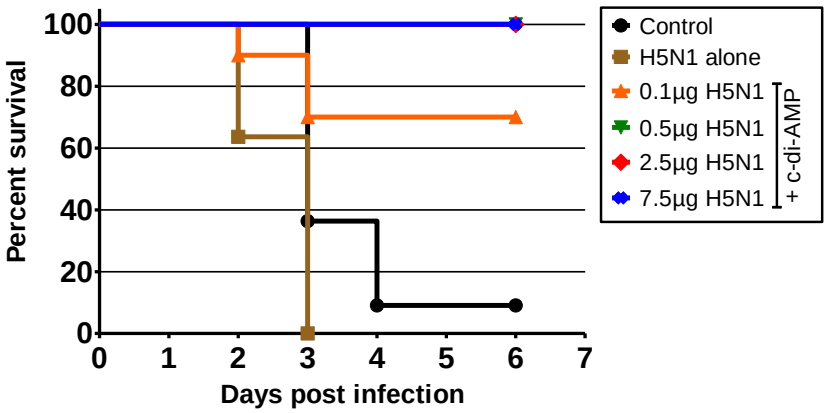

Fig. 50: Survival of immunized mice after i.n. H5N1 virus challenge. Eleven BALB/c mice per group were immunized three times with different dosages of H5N1 virosomes + c-di-AMP by i.n. route and subsequently i.n. challenged with lethal dose of the genetically modified H5N1 virus strain NIBRG-14. Animals either died or were sacrificed when their weight loss was higher than 25% respect to the starting value.

As an overall result for the immunization and challenge studies with H5N1 virosomes and c-di-AMP it has to be stated that in this model no dose dependent effects on Th polarization were observed. However, the results clearly demonstrated that vaccination with reduced Ag dose elicits strong immune responses which are protective in a challenge setting. The lack of dose dependent effects suggests that the experimental setting was suboptimal. This might be explained by two factors, (i) the strong induction of Th17 cells triggered *per se* by i.n. vaccination [142], and (ii) the stimulation of Th17 polarization by c-di-AMP [200, 201]. These two factors can lead to a situation in which Th17 polarization is already optimal and cannot be further increased by the reduction of the antigen dose.

6.3 Mechanism of Th17 differentiation blockage by NKT cells after stimulation with α-galactosylceramide

As described in the introduction, Th17 immune responses are not always beneficial for the host. Beside autoimmune and inflammatory diseases, Th17 cells have been associated with non-optimal immune responses when induced following infection with certain pathogens (see 2.1.4 and 2.3). Thus, it would be helpful not only to induce Th17 polarization, but also to shut it down at will. It is known that αGC activates NKT cells and polarizes the immune response towards the Th2 phenotype while blocking Th17 induction. However, there is a paucity of knowledge on the underlying mechanisms. The elucidation of these molecular events would facilitate the exploitation of αGC as immune modulator, as well as provide hints on potential molecular targets for immune intervention. Thus, the second part of this work was focused on dissecting the mechanisms leading to the blockage of Th17 polarization after immunization with αGC as adjuvant. For the studies presented in the following, a pegylated form of αGC (αGCPEG) was used, which shows enhanced solubility and immune stimulatory activity [193].

In the first experiment, the influence of different adjuvants co-administered with OVA via i.n. route on the elicited Th17 response was investigated. Curdlan and c-di-AMP are known to be strong inducers of Th17 cells, whereas the other used adjuvants lead to moderate IL-17 responses ([142, 201, 219, 220]). The animals were immunized and subsequently boosted on day 14 and 21. After a total of 42 days, the number of IL-17 secreting splenocytes was analyzed by performing ELISpot assays. The groups immunized with Curdlan and c-di-AMP displayed a high frequency of IL-17 producing cells, as compared to the groups where only weak Th17 polarization was detected (Fig. 51). Beside the negative control groups, only αGCPEG did not induce Th17 cells at all. The absence of Th17 cells in αGCPEG immunized mice is remarkable since i.n. immunization should always lead to increased Th17 immune responses [142].

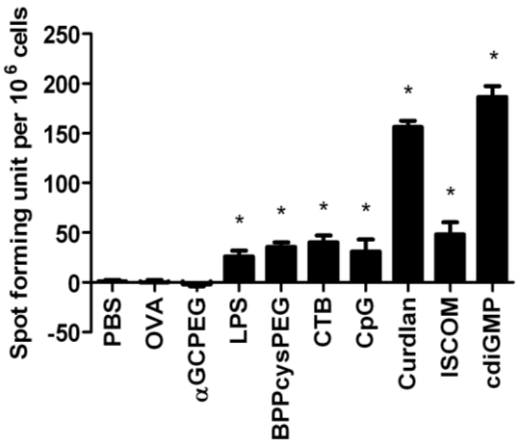

Fig. 51: αGCPEG blocks induction of Th17 immune responses after i.n. immunization. C57BL/6 mice per group were immunized with OVA co-administered with different adjuvants via i.n. route for a total of three times in 21 days and sacrificed after a total of 42 days. Splenocytes pooled for every group were restimulated with OVA in ELISpot assay to detect IL-17 immune responses. Shown are the spot forming units per 10^6 cells with subtracted background. Each bar represents the mean of 5 samples plus SEM. *, statistically significant ($p<0.05$). n=5 [221]

To investigate whether the previously described activation of NKT cells by αGCPEG is responsible for the observed effect, an *in vitro* proliferation assay with sorted naïve CFSE-labeled OTII cells and DCs was performed. The cells were stimulated with OVA-peptide AA323-339 under Th17 polarizing conditions in the presence or absence of sorted NKT cells. To the samples either αGCPEG or BPPcysPEG as control was added. After 4 days of culture cells were restimulated, stained for IL-17 and analyzed by FACS. The samples without addition of NKT cells showed a strong induction of IL-17 producing cells, independently of the added adjuvant (Fig. 52A). The addition of NKT cells led to a slight reduction of Th17 frequency in samples where BPPcysPEG was added (from 31% down to 9%). In contrast, in samples cultured in the presence of αGCPEG the polarization to Th17 cells was completely blocked. Only 0.4% of cells produced IL-17 in the presence of NKT cells, as compared to 27% in the absence of NKT cells. To clarify if this blockage was mediated by direct cell to cell interaction with NKT cells or by soluble factors released by activated NKT cells, *in vitro* proliferation assays with conditioned culture medium were performed. To OVA-peptide stimulated co-cultures of sorted $CD4^+$ naïve CFSE-labeled OTII cells and DCs supernatants from 24 h cultures of DCs plus αGCPEG, either in the presence or absence of NKT cells, were added. In samples where conditioned medium from cultures without NKT cells was added, 27% of divided $CD4^+$ T cells produced IL-17, whereas the addition of NKT co-culture supernatants led to a complete blockage of Th17 differentiation (Fig. 52B).

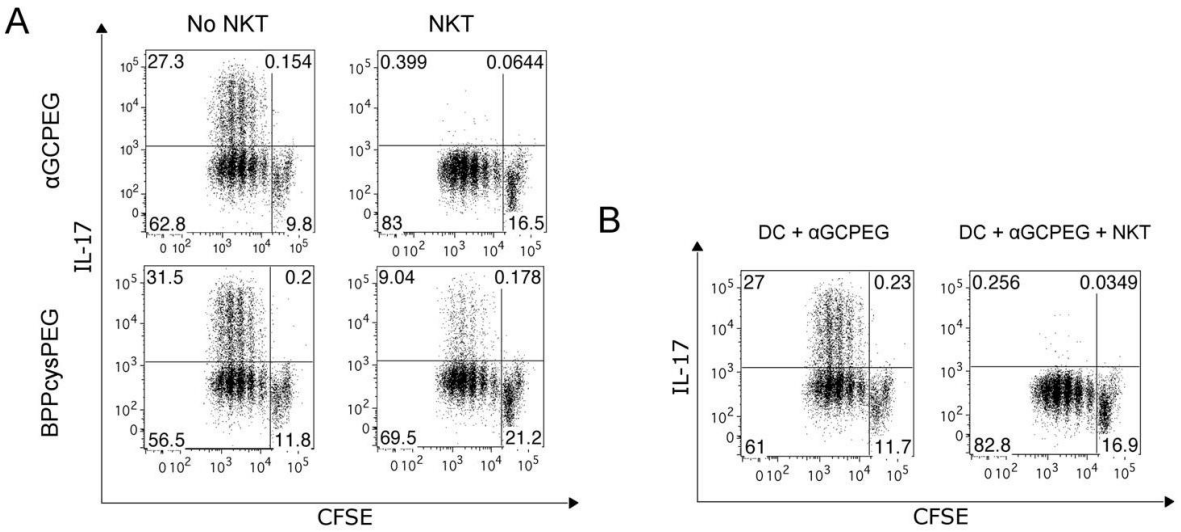

Fig. 52: NKT cells block Th17 differentiation *in vitro* by soluble factors. Sorted naïve $CD4^+$ T cells from OTII mice were labeled with CFSE and co-cultured with DCs under Th17 polarizing conditions in the presence of OVA-peptide AA323-339. Cultures were supplemented either with αGCPEG or BPPcysPEG. After 4 days cells were restimulated and analyzed for IL-17 production by FACS. (A) To some cultures sorted NKT cells were added (right panels). (B) Cultures were supplemented with supernatants collected from 24 h cultures of DCs in the presence of αGCPEG (left panel) or co-cultures of DCs and NKT cells in the presence of αGCPEG (right panel). Shown are the dot plots from representative samples for each condition. [221]

These results pointed to the fact that soluble factors released by NKT cells are responsible for the absence of Th17 cells. To identify these soluble factors, the levels of IL-4, IFNγ and IL-2 were measured by ELISA 24 h after the co-culture. As in the previously described experiments, naïve $CD4^+$ OTII cells and DCs were co-cultured either in the presence or absence of NKT cells and with the addition of αGCPEG or BPPcysPEG. Cells cultured with αGCPEG showed high levels of IL-4 and IFNγ, which were dramatically decreased when NKT cells were present (Fig. 53A). The supernatants from samples incubated with BPPcysPEG showed only low levels of IL-4, but high levels of IFNγ when NKT cells were added. No remarkable differences between the used adjuvants were observed when comparing IL-2 levels. In both cases relatively high IL-2 levels were detected and a small decrease of IL-2 concentration was observed when NKT cells were present.

Neutralizing Abs against IL-4 and IFNγ were used in subsequent experiments to check if the observed differences in these cytokine levels were responsible for the impaired Th17 differentiation. The neutralizing Abs were added single or combined to the above described *in vitro* proliferation assays, whereas no Abs were added to control wells. In the controls the induction of IL-17 producing cells was blocked by the

presence of NKT cells (Fig. 53B). This effect was weaker in the presence of αIL-4 and especially αIFNγ, which led to 1.4% or 4.3% of Th17 cells respectively. However, only the combination of both neutralizing Abs almost restored the Th17 polarization in the presence of NKT cells to the level observed in the absence of NKT cells. In summary, the presented *in vitro* experiments clearly demonstrated that the blockage of Th17 induction in the presence αGCPEG is mediated by NKT cells releasing IL-4 and IFNγ.

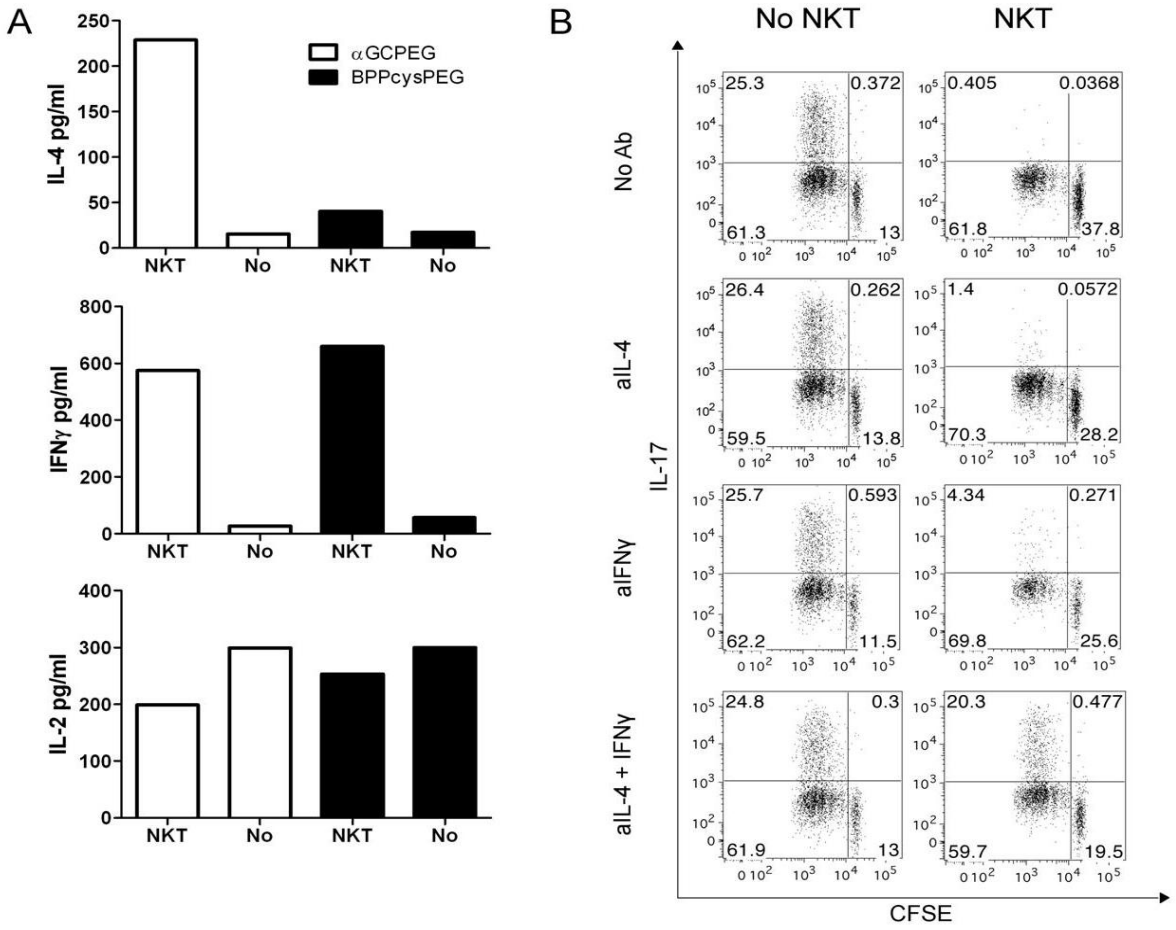

Fig. 53: NKT cells block Th17 differentiation by secretion of IL-4 and IFNγ. (A) Sorted naïve CD4$^+$ T cells from OTII mice were co-cultured with DCs under Th17 inducing conditions in the presence of OVA-peptide AA323-339. Cultures were supplemented with αGCPEG or BPPcysPEG in the presence or absence of NKT cells. Supernatants were collected after 24 h and the levels of IL-4, IFNγ and IL-2 were measured by ELISA. (B) Sorted naïve CD4$^+$ T cells from OTII mice were stained with CFSE and then co-cultured with DCs under Th17 inducing conditions in the presence of OVA-peptide AA323-339 and αGCPEG. To some cultures NKT cells (right panels) and neutralizing Abs against IL-4 and/or IFNγ were added. Cells were restimulated after 4 days and analyzed for IL-17 production by FACS. [221]

To further prove these observations, *in vivo* experiments with Jα281 KO mice which lack NKT cells were performed. C57BL/6 and Jα281 KO mice were immunized by i.n. route with OVA co-administered with αGCPEG. C57BL/6 control groups were

immunized with PBS, OVA alone or OVA plus BPPcysPEG. The animals were boosted on day 14 and sacrificed after a total of 28 days. Th17 induction in splenocytes was accessed by IL-17 ELISpot (Fig. 54). In C57BL/6 mice immunized with PBS or OVA alone almost no IL-17 positive cells were detected, whereas the co-administration of OVA and BPPcysPEG led to a moderate IL-17 response. The application of OVA in combination with αGCPEG resulted in only few IL-17 positive cells in C57BL/6 mice, but in high numbers in Jα281 KO mice. With these results, NKT cells have been shown to be responsible for αGCPEG induced blockage of Th17 polarization also *in vivo*.

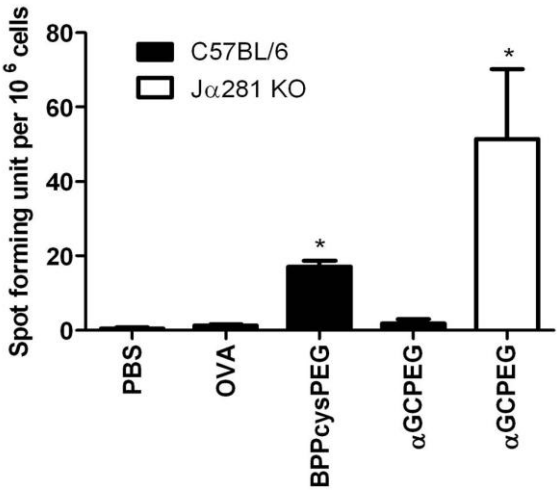

Fig. 54: NKT cells block Th17 differentiation after i.n. immunization *in vivo*. Five C57BL/6 or Jα281 KO mice per group were immunized with OVA plus αGCPEG via i.n. route and boosted after 14 days. Control groups received PBS, OVA alone or OVA plus BPPcycPEG. After a total of 35 days animals were sacrificed and IL-17 ELISpot with group pooled splenocytes was performed. Shown are the spot forming units per 10^6 cells with substracted background. Each bar represents the mean of 5 samples plus SEM. *, statistically significant ($p<0.05$). [221]

Based on the described results the interesting question came up, if the αGCPEG mediated blockage of Th17 cells caused by NKT cells can be used to attenuate strong Th17 immune responses when co-administering αGCPEG with a Th17 polarizing adjuvant. To answer this question, C57BL/6 mice were immunized with OVA co-administered with αGCPEG, LPS, Curdlan or combinations of these immune stimulators. The animals were boosted on day 14 and 21 and sacrificed after a total of 42 days. The Th17 immune response was measured by IL-17 ELISpot from group pooled splenocytes. Control groups immunized with PBS or OVA alone showed no cells positive for IL-17 and also the co-administration of OVA and αGCPEG did not lead to a Th17 induction. The immunization with OVA plus LPS induced a moderate

IL-17 signal, which was significantly reduced when αGCPEG was added to the formulation (Fig. 55). The same dampening effect was observed when αGCPEG was co-administered with Curdlan. The very strong Th17 immune response induced by Curdlan alone was reduced to a moderate level. These observations gave evidence that αGCPEG is a promising candidate immune modulator, since it cannot only completely block Th17 induction when administered as adjuvant, but also fine tune the immune response when co-administered with other adjuvants.

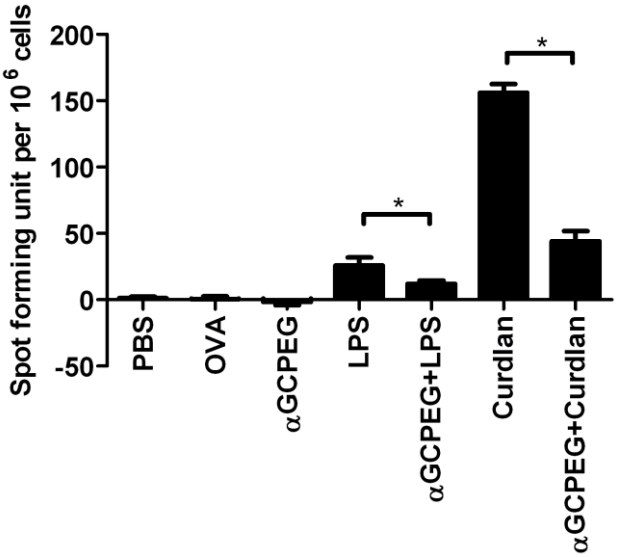

Fig. 55: αGCPEG efficiently modulates the effect of other adjuvants. Five C57BL/6 mice per group were immunized with OVA co-administered with different combinations of LPS, Curdlan and αGCPEG via i.n. route and boosted 2 times. After a total of 42 days animals were sacrificed and IL-17 ELISpot with group pooled splenocytes was performed. Shown are the spot forming units per 10^6 cells with subtracted background. Each bar represents the mean of 5 samples plus SEM. *, statistically significant ($p<0.05$). [221]

To sum up, this study identified the release of IL-4 and IFNγ by αGCPEG stimulated NKT as the underlying mechanism of Th17 blockage when administering αGCPEG as adjuvant. We further demonstrated that αGCPEG cannot only completely shut down Th17 immune responses, but even fine-tune Th17 induction when co-administered with other adjuvants.

7 Discussion

Vaccines should promote strong immune responses, including a robust immunological memory, thereby protecting against potential future encounters with pathogens. Currently available vaccines mostly trigger Th2 dominated immune responses with poor induction of CTLs [222, 223]. Thus, vaccine development efforts were focused on the development of tools and strategies to promote efficient Th1 and CTL responses [165, 167, 173, 186, 222]. Following the recent discovery of Th17 cells, the understanding of the role played by this T cell subset in chronic inflammatory and autoimmune diseases was one major aim of research. In fact, during the development and testing of modern vaccine, Th17 cell induction has not been truly considered or was even disregarded. Nevertheless, a considerable body of experimental and clinical evidence strongly supports a prominent role of Th17 cells during infection. For example, the importance of inducing Th17 responses for efficient clearance has been lately shown for pathogens as *Candida albicans*, *Klebsiella pneumoniae* or the influenza virus [116, 123-125], whereas Th17 induction might be harmful during *Borrelia* infection [224].

It is important to consider that the induction of the inappropriate Th phenotype against a certain pathogen following vaccination can lead to higher susceptibility, severe health problems or even death. While BALB/c mice are unable to control a *Leishmania major* infection due to stimulation of a Th2 dominated immune response, C57BL/6 mice mount a protective Th1 response (see also section 2.1.3). For Th1 and Th2 cells it was already described under which conditions (*e.g.* Ag dosage and cytokine milieu) their differentiation is induced [67-69, 82, 83]. In contrast, our knowledge about Th17 cells and their induction is rather fragmentary. For example, Purvis and co-workers recently showed the induction of Th17 cells at low TCR stimulation [151]. However, the results of this study are in contrast to other studies reporting Th17 induction following strong TCR stimulation [149, 150]. So far, no explanation was found for these contradictory results. One reason might be that different experimental models and methods were used in these studies. The study that showed Th17 polarization as a result of low TCR stimulation was performed mostly using human cells stimulated with either αCD3 Ab or super antigen, and it was based only on *in vitro* results. Both stimulation methods differ from the natural

process of TCR binding to peptide Ags presented by MHC class II molecules. This can in turn lead to abnormal T cell behavior, due to the triggering of unusual signaling pathways [152]. Accordingly, it has still to be proven if the results of this study are reproducible using a peptide Ag and/or performing *in vivo* experiments. In contrast, the need of a strong stimulus for Th17 induction described by Gomez-Rodriguez *et al.* and Iezzi *et al.* was observed in the murine system. While Gomez-Rodriguez performed only *in vitro* experiments using αCD3 Ab for stimulation, Iezzi performed both *in vitro* studies using only TCR transgenic cells stimulated by the corresponding Ag and *in vivo* experiments using a very limited range of Ag concentrations. However, the elicitation of strong Th17 immune responses following strong stimulation is not in agreement with the strong Th17 immune responses always obtained after i.n. application of the Ag. In fact, i.n. administration is normally linked to a poor Ag uptake, thereby leading to a reduction of the active Ag dose [142].

In this work, *in vitro* experiments were combined with *in vivo* adoptive transfers and immunization experiments using a total of three different TCR transgenic and two wild type mouse strains to get broader and deeper insights into the polarization of Th17 cells depending on the stimulation strength. In addition, the influence of TCR affinity to Ags presented by MHC class II on Th polarization was also taken under account, since in this regard no experimental data were available until now. The results discussed in detail in the following give direct answers to some of the pending questions and contribute to a better understanding of Th polarization process. For validation of the new findings, low dose immunizations using H5N1 virosomes followed by viral challenge were performed. Since not only the induction but also the controlled blockage of Th17 cells is interesting for vaccinations, the last part of this thesis was focused on the mechanism of Th17 blockage by αGCPEG.

7.1 Ag dose dependent Th17 induction differs in DO11.10 and OTII mice

In the present work, DCs and T cells were incubated for the whole *in vitro* proliferation assay in the presence of the Ag. This procedure led to more robust data as compared to those obtained when DCs were pulsed with Ag only for 3 h. Most probably, the washing steps following DC pulsing were the reasons for the observed variations between the single experiments as strong variations in the DC numbers

were observed after the washing. As demonstrated by experiments under controlled conditions, ratios the DC to T cell ratio was crucial for the final outcome (Fig. 27-Fig. 29). The Ag dose dependent induction pattern of Th1 and Th2 polarization for cells from DO11.10 and OTII mice demonstrated that these two mouse strains mount similar Th responses. In most experiments Th1 as well as Th2 cells were induced preferentially at low to intermediate Ag dosages, which is in contrast to what was published before [82]. Constant *et al.* described Th1 polarization at intermediate to high Ag doses, whereas Th2 cells were observed at low dosages [82]. Despite differences in the experimental model and range of Ag used, one potential explanation is that the starting T cell population was less pure and characterized in the previous study as a result of the technical limitations in 1995. Therefore, it cannot be excluded that pre-activated or memory cells against different Ag were used for the experiments. It is known that memory cells can be activated independently of the presence of their specific Ag by cytokines, especially IL-2 [225].

Since in the present work only minor differences between the two mouse strains were observed in terms of Th1 and Th2 induction, the fundamental difference in Ag dose dependent induction of Th17 cells in these strains was quite surprising. DO11.10 cells differentiated to Th17 phenotype in the presence of low or high Ag concentrations, whereas OTII cells showed a peak of Th17 induction only at high Ag doses. The observed polarization towards Th17 phenotype at high doses in both strains is in line with the two studies named before, especially when only the Ag concentration range used in the study by Iezzi *et al.* is taken under consideration. There, cells were stimulated with peptide concentrations between 10 and 10000 nM. However, in the recent work the low dose peak detected for DO11.10 cells was elicited by Ag concentrations lower than 10 nM. Thus, it is possible that this observation was missed by Iezzi *et al*., since they did not titrate the Ag dose low enough in their experiments. However, this does not explain the differences observed between the mouse strains. Thus, further experiments to investigate the impact of culture conditions and OVA-peptide affinity of DCs were performed to understand this phenomenon. As described in the introduction (2.1.4), the addition of IL-6 and TGFβ to cultures induces enhanced Th17 polarization. These Th17 inducing conditions especially increased the Th17 polarization at intermediate and high Ag doses in both strains. Therefore, the low dose peak of Th17 polarization observed in DO11.10 cells

under normal conditions (full RPMI medium without addition of cytokines) was in most experiments very weak as compared to the high dose peak when Th17 inducing conditions were used. The strongest change of Th17 levels at high Ag concentrations was also observed in the study of Iezzi *et al.* when Th17 polarizing cytokines were added. An Ag dose dependent influence of polarizing cytokines was already reported in the development of Tregs [35]. The same was true for the absence of Th1 and Th2 cells under Th17 polarizing conditions [226].

Like the addition of polarizing cytokines, the neutralization of IL-2 alone or in combination with neutralization of IL-4 and IFNγ also resulted in a similar enhancing effect on Th17 polarization. IL-2 has been shown to be essential for Th1 and Th2 development, while it blocks Th17 differentiation [91, 227, 228]. Therefore, neutralization of IL-2 enhances Th17 polarization directly, as well as indirectly by inhibiting Th1 and Th2 polarization. This last effect is mediated by decreased levels of IL-4 and IFNγ in the culture, which further facilitates Th17 induction. However, in both investigated mouse strains the general Ag dose dependent Th17 induction pattern did not change by the addition of neutralizing Abs. Thus, different levels of these cytokines could be excluded as reason for the observed differences under normal conditions. The increased Th17 polarization when Th17 inducing cytokines were combined with neutralizing Abs in the assays were observed in both strains only at intermediate to high Ag doses, but not at low dosages. As consequence, it can be hypothesized that highly polarizing conditions are only effective if at least intermediate Ag concentrations are present. In which extend these rather artificial conditions also occur *in vivo,* still needs to be investigated.

It is known that also IFNβ can influence Th polarization and that BMDCs generated by the addition of GM-CSF are able to produce this cytokine [229, 230]. However, neutralization of IFNβ in the assays did not result in any effect, neither under normal nor under Th17 inducing conditions. This might be explained by low levels of IFNβ in the cultures, which were not measured during the experiments.

As discussed before, it was determined that the DC to T cell ratio plays an important role in Th differentiation. Higher numbers of DCs can present more Ag and provide increased numbers of co-stimulatory molecules to T cells, which resulted in an

increased Th17 polarization under normal, but not under Th17 inducing conditions, at intermediate and high Ag concentrations. In experiments for this thesis with different DC numbers, an increase over the 1 to 5 ratio of DCs to T cells resulted only in a small increase of Th17 cells. This might be explained by a study from Hopken and co-workers where it was shown that beginning with ratios of 1 to 2, the T cells stopped to proliferate and switched to a tolerant state [231]. One reason for the absence of an effect under Th17 polarizing conditions might be the presence of a threshold in Th17 numbers that once reached could not be raised by a further increase of the DC numbers. This would be in line with the observation that the Th17 frequency reached a maximum at 1235nM OVA-peptide under Th17 polarizing conditions. The influence of co-stimulatory molecules on Th17 differentiation was the target of intense investigation in the last years. It was reported that strong interaction between CD40/CD40L at high Ag doses mediates IL-6 secretion by DCs, thereby enhancing Th17 polarization [145, 150]. In addition, also for B7/ICOS, OX40/OX40L and TIM-1/TIM-4 interactions a stimulating effect on Th17 induction has been shown [232-236]. The interactions between CTLA-4/B7 and PD-1/PD-1L instead counteract Th17 polarization [236-238]. Controversial results have been published concerning the role of CD28/B7 interaction. While two studies reported the essential need of this co-stimulation for Th17 induction, other studies showed a negative effect on Th17 polarization [107, 141, 236, 239, 240].

By the use of a third TCR transgenic mouse line it was demonstrated that Th17 polarization at low Ag concentrations is not only a special phenomenon for DO11.10 cells. Moreover, it was shown that Ag dose dependent Th1 and Th2 polarization of TCR-HA cells behaves similarly to what was reported by Constant *et al.* for their model [82]. In contrast to DO11.10 and OTII cells, Th17 inducing conditions increased Th17 polarization of TCR-HA cells exclusively in the presence of low Ag doses. This effect might be explained by a different affinity of the TCR or MHC class II molecule to the Ag, which would result in altered stimulation strength.

The similar loading of OVA-peptide on BMDCs derived from BALB/c and C57BL/6 demonstrated by using FITC-labeled OVA-peptide rules out a differential affinity of MHC class molecules from both strains as a reason for the observed differences between DO11.10 and OTII mice. Therefore, it is most likely that this phenomenon is

caused by different affinity of the TCRs to the OVA-peptide. This hypothesis has been explored by using of sequence altered OVA-peptides, but the obtained results were not conclusive. As previously described, alterations of AA position 335 and 336 led to decreased proliferation rates of DO11.10 and OTII cells [215]. Unfortunately, the proliferation was that dramatically reduced, making it impossible to identify an effect of Th subsets.

In summary, for each TCR transgenic mouse strain robust results for the Ag dose dependent Th17 induction were achieved. Beside some similarities, evident differences in Th17 polarization where observed between the mouse strains, which were most probably based on differential TCR affinity to the corresponding peptide Ag. These findings show that experiments performed with different TCR transgenic mouse models cannot always be extrapolated to other systems. This might explain some contradictory observations in published studies using different mouse models. To expand these observations, studies were performed in which differential affinity of the Ag to the TCR was ruled out by direct CD3 stimulation with αCD3 Ab. This method also allowed to study cells from wild type mice.

7.2 Ag independent stimulation with αCD3 Ab induces Th17 polarization at low dose

Näive $CD4^+$ T cells from BALB/c and C57BL/6 wild type mice as well as from OTII mice were stimulated *in vitro* with different concentrations of αCD3 Ab to study Ag independent Th17 polarization. The cells from all three mouse strains differentiated especially at low αCD3 Ab concentrations towards the Th17 phenotype. Under Th17 inducing conditions the highest Th17 numbers were detected at low αCD3 Ab concentrations. These findings clearly demonstrated that (i) there are no general differences between BALB/c and C57BL/6 concerning Th17 polarization, and (ii) a Th17 polarization is induced when the strength of stimulation is low. The observation of Th17 polarization in the presence of low αCD3 Ab concentrations is in line with recent findings in the human system [151]. In that study Th17 induction was also only observed when low αCD3 Ab concentrations where used for stimulation. Nevertheless, it has to be mentioned that a study published by Gomez-Rodriguez *et al.* showed induction of Th17 polarization only with high αCD3 Ab concentrations

[140]. A possible explanation for this contradiction might be the use of different protocols. While in the recent work RPMI medium was used, Gomez-Rodriguez *et al.* cultured their cells in IMDM medium. It has been reported that IMDM medium contains high amounts of aryl hydrocarbon receptor agonists which induce Th17 polarization. It is possible that under these polarizing conditions Th17 induction only occurred in the presence of high stimulus concentrations. In addition, we observed similar effects when neutralizing Abs for IL-2, IL-4 and IFNγ were combined with Th17 inducing conditions (7.1).

The observation that cells from wild type and OTII mice were polarized towards the Th17 phenotype only in the presence of low αCD3 Ab concentrations gives further evidence that the observed differences in Ag dose dependent Th17 induction between DO11.10 and OTII mice are based on different affinities of their TCRs to the OVA-peptide AA323-339. As a consequence of these results it has to be reconsidered to which extent and under which specific experimental conditions TCR transgenic mouse models are a valid tool to extrapolate to the natural *in vivo* situation.

7.3 Adoptive transfer of TCR transgenic T cells to wild type recipient mice as a model to study TCR transgenic cells *in vivo*

The adoptive transfer is a well-established tool for characterization of T cell activation *in vivo,* since TCR-transgenic mice are tolerogenic to their TCR specific Ag (reviewed in [241]). In the recent study adoptive transfer experiments were performed to analyze antigen dose dependent Th polarization of OTII cells in an *in vivo* setting, in comparison to the *in vitro* studies performed before. Intra footpad immunization and subsequent analysis of the triggered immune responses in pLNs 3 days after injection appealed as the most suitable technique to assess proliferation and cytokine production of the transferred cells. Therefore, it was rather unexpected that the animals of each group showed extreme variations in cytokine production when immunized with different dosages of OVA co-administered with Curdlan. Since the recovery and proliferation rates of transferred cells were comparable between the mice of each group, inaccurate injection of OTII cells or immunization solution can be excluded as a possible explanation for this high variability. One problem might have

been the use of the glucan molecule Curdlan as adjuvant, which is not soluble in water. Therefore, injection solutions containing Curdlan have to be well shaken and quickly injected to guarantee homogenous distribution of the adjuvant. It cannot be ruled out that the animals of each group received different amounts of Curdlan. It is important to take into account that the concentration of adjuvant directly influences Th polarization, as discussed in the next section (7.4). This means that for a constant read out, it would be favorable to repeat this experiment with larger group sizes or to use a water-soluble adjuvant (*e.g.* pegylation of Curdlan). Due to limitations of the donor mice numbers it was not possible to test this new approach.

7.4 Immunization studies with OVA-peptide reveals Th17 polarization at low Ag dose

To evaluate the efficacy of new vaccination strategies, experimental small animal models constitute a commonly used approach. However, until now only one study investigated Ag dose dependent polarization of Th17 cells using an *in vivo* mouse model [150]. In that study C57BL/6 mice were immunized with 20, 50 or 100 µg of glycoprotein (gp; AA61-81) of the *Lymphocytic Choriomeningitis Virus* (LCMV) co-administered with CFA via s.c. route. While in mice immunized with 50 and 100 µg of Ag high numbers of Th17 cells were detected, almost no Th17 response has been observed in animals that received only 20 µg of peptide. Interestingly, when similar studies were performed during this thesis using OVA, robust Ag specific Th17 responses were induced in mice immunized with low doses of OVA, independently of the application route. The observed differences might be explained by the different experimental design. In the present work mice were immunized using full-length OVA protein (*i.e.* mimicking normal conditions in the field following vaccination), whereas Iezzi *et al.* used a peptide Ag. Proteins are ingested and processed by APCs, which results in the formation of different peptide Ags that are presented on the cell surface in the context of MHC molecules [1]. In contrast, peptide Ags can directly bind to empty MHC class II molecules on the surface of APCs [242]. By this means, the nature of Ag influences the triggered signaling cascades in APCs, as well as their activation status. This leads to different stimulatory properties and, thereby, to different adaptive immune responses. Furthermore, it was reported for OVA that most T cell responses are elicited by the peptide AA323-339 [241, 243, 244]. Thus, OVA-

peptide AA323-339 only adds to 4% of the total molecular weight of OVA and the number of stimulating moieties strongly differs when comparing full-length protein and peptide. Since Iezzi *et al.* used 20 µg of gp peptide as their “low” dosage it is possible that this dosage still provided strong stimulation. This might explain the observed differences.

Another point to mention is the observed influence of the LPS dose on Th17 polarization. High LPS concentrations co-administered with a medium OVA dose elicited similar Th17 polarization as low dose Ag co-administered with low dosages of LPS. DCs are activated by LPS, which in turn leads to up regulation of co-stimulatory molecules that often favors Th17 polarization, as discussed before (7.1). Interactions of Ag dose dependent effects and co-stimulation have been described for Th1 and Th2 cells [245]. In that study strong co-stimulation induced Th1 and Th2 responses only in the presence of high and low Ag doses, respectively. However, the observation of the present thesis that Th17 induction at higher Ag doses is dependent on high co-stimulation has not been reported before.

Taking together the results obtained from *in vitro* and *in vivo* experiments, the most probable reason for the differences in Ag dose dependent Th17 polarization between DO11.10 and OTII mice is the existence of differences in the affinity of the TCR to the OVA-peptide AA323-339. Thus, TCR independent stimulation by αCD3 Ab as well as the immunization studies showed a preferentially induction of Th17 cells when low stimulation strength was applied. Another fact is that highly Th17 polarizing conditions, especially at high Ag doses induced strong Th17 polarization, whereas at low Ag doses almost no effect was observed. This clearly demonstrated that Th17 polarizing conditions are Ag dose dependent. The observation that high LPS concentrations co-administered with high Ag dosage also polarized towards the Th17 phenotype *in vivo* contributes to this finding. As a conclusion it can be formulated that Th17 polarization seems to be induced either by low stimulation strength in the presence of poor Th17 polarizing conditions or by high stimulation strength in the presence of strongly polarizing conditions (Fig. 56).

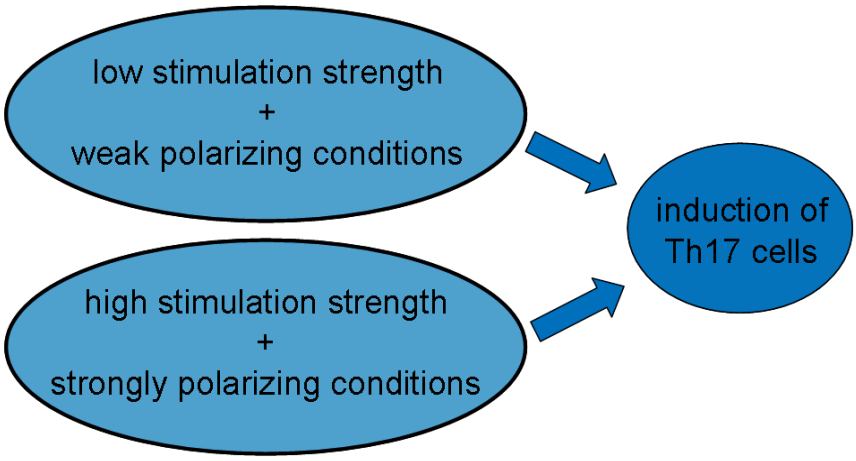

Fig. 56: Hypothetical model of Th17 induction depending on stimulation strength and polarizing conditions.

This two component model would explain the contradictory results published before and could help to better control the induction of Th17 cells. Until now, all discussed experiments were performed with the model Ag OVA or αCD3 Ab. To validate if the obtained results can be exploited to control Th17 induction in a clinical setting, vaccination studies were performed using a vaccine formulation against influenza.

7.5 Mice immunized with low H5N1 virosome doses show a Th1/Th17 immune response protective against viral influenza challenge

Despite several improvements in health care, infectious diseases still represent one of the major health problems worldwide. For example, one of the threats to mankind all over the world is the influenza virus. We are faced with two different settings of influenza occurrence: (i) the seasonal influenza which spreads every year in autumn/winter and (ii) the spontaneous unpredictable outbreaks of pandemic influenza. In case of seasonal influenza, viruses of the subtype class A or B spread which are especially dangerous for newborns, elderly and immune-compromised people [246, 247]. Although vaccines against influenza are available since the 1940s, between 1985 and 2009 in average almost 11,000 patients per year died in Germany due to influenza infections [248-250]. Worldwide 250,000 to 500,000 people are killed every year by seasonal influenza outbreaks [247]. The World Health Organization (WHO) established the “Global Influenza Surveillance Network (GISN)” which earmarks every year three influenza strains for incorporation in the next vaccine

formulation. The seasonal influenza vaccine is composed of two influenza A strains (H1N1 and H3N2) and one influenza B strain. The exact strains are chosen as a prediction of the upcoming flu season based on isolates from the season before [247]. This system of prediction has one major drawback as well as one major advantage. If the prediction by the GISN was wrong, the vaccine will provide only limited protection. Thus, a meta-analysis of 31 independent studies about seasonal influenza vaccines revealed an overall efficacy of 67-75%, whereas in some seasons the efficacy of vaccines dropped to 35% [246]. However, the advantage of the system is that the prediction of the most probable strains for the next season allows the industry to produce the vaccines in advance and to supply them to people of risk groups in sufficient amount before the flu season starts. Since the 1950s, influenza vaccines are produced by inoculating chicken eggs with the vaccination virus strains, which is a strongly time consuming process. Cell culture systems for virus production are under development but are not fully approved yet [251]. Nevertheless, vaccine production capacities are very limited, in 2008 the worldwide capacity for seasonal influenza vaccine production amounted to approximately 820 million doses [252]. Production time and capacities would be also one of the major problems in a spontaneous pandemic influenza outbreak since no vaccine can be produced in advance as it is the case with seasonal influenza. Calculations for the H1N1 pandemic in 2009 came to the result, that it would take more than one year to produce 5 billion doses of a new vaccine, not taking under account the time needed to identify the virus strain and to develop the complete vaccine formulation [253]. To shorten the time needed for the development of such a vaccine and its approval by the authorities, industry and authorities developed the so called “mock-up” and “emergency” procedure for the case of a pandemic influenza outbreak. In this mock-up procedure, a vaccine formulation with a virus strain that potentially cause a pandemic is developed and approved in advance. In case of a pandemic influenza outbreak, the virus strain of the already tested and approved mock-up vaccine is exchanged against the pandemic strain so that the final vaccine can be produced and used after short safety tests. In contrast, the emergency procedure applies after a pandemic has already been declared and shortens the approval time by authorities to about 2 month instead of the normal 7 month. A third way to facilitate the supply with a pandemic vaccine is the modification of an already approved seasonal influenza vaccine [254]. So far, all the described scenarios are based on the use of

full live-attenuated or inactivated/split viruses as Ag in the vaccine formulations whose production is time consuming. An alternative approach which would be faster and more elegant constitutes the use of virosomes as vaccine Ag [207]. For the production of virosomes only purified HA and NA molecules are necessary, which can be isolated from whole virus or fast and easy produced by recombinant expression systems [205, 255]. Finally, another approach to speed up the vaccine production is the reduction of needed Ag per vaccination dose, the so called dose sparing. This can be achieved by the addition of a powerful adjuvant to the formulation which stimulates the immune system and/or enhances Ag uptake and presentation [200, 256, 257].

As described in the introduction (2.1.3), vaccinations need to stimulate the appropriate Th phenotype for each pathogen in order to provide a protective immune response. In case of the influenza virus it is not yet fully elucidated which Th phenotype is needed. Adoptive transfer studies from S. Swain and colleagues proved that influenza specific $CD4^+$ effector cells are sufficient to protect against otherwise lethal viral challenge. Protection was achieved when either an unsorted pool of influenza specific $CD4^+$ effector or sorted influenza specific Th1 cells were transferred [258]. However, these experiments were performed in 2004, when Th17 cells where not known yet. Thus, a potential role of Th17 cells was not investigated in this study, which demonstrated the need of Th1 cells for protection against influenza. More recent studies showed that beside Th1 induction, protective immune responses included also Th17 polarization [125, 259-261]. On the other hand, for H1N1 it was reported that IL17R KO mice were better protected against viral challenge and that high Th17 cell numbers were detected in patients with severe pathology after infection with the pandemic H1N1 virus in 2009 [262, 263]. Thus, the role of Th17 cells in protective immune responses against influenza infections has to be investigated in more detail.

The initial studies carried out in this thesis demonstrated that immunization with low doses of the model Ag OVA favors Th17 polarization. Thus, in the second part of this work it was investigated whether Ag dose dependent Th17 polarization can be also achieved by following a mucosal vaccination strategy using a pathogen relevant Ag, with or without co-administration of an adjuvant. Since influenza vaccines are a good

candidate for i.n. application and the role of Th17 cells in protective immune responses against the influenza virus needed further investigation, H5N1 virosomes were chosen as Ag. The protectiveness of the elicited immune response was tested by a subsequent viral challenge. As shown in the result section, also immunization with the lower dosages of H5N1 virosomes co-administered with c-di-AMP via i.n. route led to the induction of Ag specific Th1 cells and elicited strong Th17 polarization. This resulted in protection against challenge with an otherwise lethal viral dose.

The strong Ag specific proliferation of splenocytes derived from animals immunized with 0.5, 2.5 or 7.5 µg of HA equivalents of H5N1 virosomes co-administered with c-di-AMP indicates a robust systemic induction of H5N1 specific cells by the used immunization protocol. This is in line with published studies showing the stimulation of local as well as systemic immune responses after i.n. vaccination, especially when c-di nucleotides are used as adjuvant [142, 201]. The analysis of the elicited Th subsets by ELISpot and FACS revealed a mixed Th1/Th17 dominated immune response, whereas Th2 cells were almost absent. These findings are in line with a recent study showing a Th1/Th17 dominated immune response following immunization with a trivalent influenza vaccine [261]. However, while in the ELISpot assays the number of IFNγ positive cells was slightly higher than the number of IL-17 positive cells, FACS analysis revealed a 2-fold higher frequency of IL-17 positive cells, as compared to that of IFNγ positive cells. Furthermore, with the second method also in the samples from animals immunized with 0.1 µg H5N1 virosomes co-administrated with adjuvant a clear Th17 polarization was detected, which was absent in the ELISpot assays. These differences could be explained by the fact that IFNγ can be produced not only by Th1 cells, but also by $CD8^+$ T cells, NK cells, NKT cells, macrophages and DCs [221, 264-266]. Thus, with an ELISpot assay it is not possible to identify the source of the detected IFNγ, since only already secreted cytokines are measured. In contrast, when performing the multi-parametric FACS analysis only $CD3^+$ $CD4^+$ $CD8^-$ cells were analyzed for cytokine expression. In addition, by ELISpot cytokines produced by more than one cell can be detected as a “single” spot. Therefore, while the FACS analysis presents the “true” picture of the Th1 population induced following vaccination, the IFNγ signals detected by ELISpot analysis most probably derived also from non-Th cells. This in turn might explain the

reduced numbers of IFNγ producing cells observed by the FACS analysis. However, the elicited Th1 response was robust, and able to confer protection against a lethal influenza infection.

A possible explanation for the differences observed in mice immunized with lowest dosage of virosomes when comparing ELISpot and FACS data could be related to the different principles of signal measurement. ELISpot assays allow just the detection of cytokines already secreted, whereas FACS in addition allows the detection of not yet secreted cytokines. Most probably the lowest Ag dose only led to cytokine production but not secretion, as some kind of short term non secreted cytokine storage, like previously described [267-269]. The physiological role of this phenomenon is not known yet. However, as discussed later, the immune response triggered by 0.1 µg of virosomes was not able to protect all mice against viral challenge.

Comparing the results obtained after immunizations with either OVA or H5N1 virosomes as Ag, it has to be stated that the strongly increased induction of Th17 phenotype at low Ag dose, as compared to medium and high Ag doses was only observed when mice were immunized with OVA. The use of virosomes as Ag stimulated very high numbers of Th17 cells at all used concentrations when co-administered with adjuvant. However, it is unlikely that the observed phenomenon of low Ag dose Th17 induction is exclusively triggered by OVA, since *in vitro* proliferation assays with HA-peptide as Ag clearly demonstrated an increased induction of Th17 cells at low Ag concentrations. This finding was further confirmed when T cells were stimulated *in vitro* in an Ag independent manner by titrating αCD3 Ab, thereby resulting in a Th17 polarization at low concentrations. More likely, the differences in Th17 polarization are based on the immunization strategy and/or the vaccine formulation.

The immunizations with OVA were performed by s.c. or i.p. route, which do not favor the induction of a specific Th phenotype. Furthermore, the co-administration of LPS only leads to moderate Th17 responses by itself. In contrast, i.n. application and c-di-AMP strongly favor the induction of Th17 cells [142, 200, 201, 207, 221]. Therefore, immunization of mice with H5N1+c-di-AMP by i.n. route stimulated about 4 times higher numbers of IL-17 secreting splenocytes, as compared to those observed

following vaccination of mice using OVA+LPS (400 and 100 IL-17 positive cells per $1x10^6$ cells, respectively). Although very strong Th17 responses were elicited, the health status of immunized mice was not affected (*e.g.* neither weight loss nor scrubby hair was observed). This is important since in patients with severe cases of H1N1infection outbalanced Th17 responses were observed that led to a cytokine storm with subsequent lung damage and respiratory failure [262, 270].

When co-administering medium concentrations of OVA with very high LPS doses, very strong Th17 responses were elicited. It is possible that the used dosage of c-di-AMP co-administered with H5N1 virosomes had a comparable effect and was therefore masking a putative dose dependent effect on Th17 polarization. Another possibility could be that by the combination of Th17 inducing components in association with the i.n. application route some kind of physiological threshold of Th17 polarization was already reached at medium Ag dosages, which could not be breached by lowering the Ag dose, which would under normal conditions lead to a further increase of Th17 induction. If such a physiological threshold exists, it would be most probably controlled by Tregs, which are known to limit and balance Th17 immune responses. This special ability of Tregs is already used for therapy of Th17 linked inflammatory and auto-immune diseases [271-274]. To validate if this hypothesis explains the observed missing of increased Th17 polarization at low H5N1 doses, immunizations with different adjuvants and different application routes should be performed. Also the comparison of Treg populations after the two described immunization settings could provide new insights into Th17 biology.

However, the characterization of the elicited Th phenotypes is not sufficient to judge the effectiveness of an immunization, since it has been reported for many pathogens (*e.g. Mycobacterium* ssp., *Leishmania* ssp. and influenza virus) that a highly protective immune response depends on so-called polyfunctional $CD4^+$ T cells. These cells are characterized by their ability to secrete different combinations of the cytokines IL-2, IFNγ and TNFα and therefore play a vital role in orchestrating the adaptive immune response [48, 259, 275, 276]. The identification of these cells is only possible by multi-parametric flow cytometry, since the cytokines produced by a single cell have to be characterized simultaneously. The 9-color staining panel developed during this thesis was able to fulfill this task. Thus, in the present work it

was demonstrated that immunization with H5N1 virosomes co-administered with c-di-AMP by i.n. route was able to induce polyfunctional T cells (Fig. 49). The population producing two cytokines was clearly dominated by TNFα and IL-2 double positive cells, whereas the other combinations where only present in minor fractions. These results are in line with recent findings showing a comparable distribution of polyfunctional T cells in mice immunized with purified influenza proteins co-administered with c-di-GMP, another cyclic di-nucleotide exhibiting adjuvant properties [259]. Nevertheless, it has to be stated that the frequency of cells producing all three cytokines was lower in the present study, as compared to the results from Madhun *et al.* [259]. However, the number of polyfunctional T cells induced by H5N1 virosomes + c-di-AMP was sufficient to provide a protective immune response. Interestingly also in the group immunized with 0.1 µg virosomes and c-di-AMP polyfunctional T cells were detected.

The subsequent challenge with a lethal dose of the influenza virus revealed that the immune response elicited by the lowest vaccination dose of H5N1 virosomes (0.1 µg) was sufficient to protect only 70% of the mice. This is in line with the observed low cellular responses and absence of cytokine secreting cells, indicating that the lowest Ag dose was not sufficient to trigger robust immune responses. In contrast, all vaccine formulations encompassing higher virosome dosages co-administered with c-di-AMP induced 100% protection against viral infection. The fact that already 0.5 µg of virosomes co-administered with c-di-AMP via i.n. route are sufficient to elicit a protective immune response are remarkable in the context of dose sparing, since recent studies reported the need of 5-7.5 µg H5N1 virosomes to induce robust immune responses following vaccination [277, 278]. Thus, the here presented combination of immunization strategy and vaccine formulation allows to reduce the amount of Ag needed to elicit full protective immunity by the factor 10 to 15, as compared to similar studies. This in turn would dramatically reduce the production time of sufficient lots of a vaccine in a pandemic influenza outbreak.

In conclusion, it was demonstrated that by the combination of mucosal application route, a promising adjuvant candidate and low Ag dose immunization mixed Th1/Th17 immune responses were stimulated that were protective against a subsequent viral challenge. The strong Th17 responses which were elicited had no

negative consequences for the immunized animals. In fact, it seems that the Th17 response rather contribute to the overall protective effect. However, it has to be investigated whether the described approach can be transferred to humans still. Nevertheless, the perspectives are quite promising as the single components of the presented immunization strategy are already approved for the use in humans (i.n. application [279]), have been tested in clinical trials (H5N1 virosomes [207]) or have been shown to stimulate not only murine but also human cells *in vitro* (c-di-AMP [201]). Concerning the potential risk of neurological side effects (Bell's palsy) following i.n. vaccination, it is important to highlight that this was only seen in association with the use of AB moiety toxins and their derivatives as adjuvants (*i.e.* adjuvants for which specific receptors exist in neurons [280, 281]). Thus, the use of c-di-AMP as i.n. adjuvant might not represent any risk. In addition, we have assessed the activity of the adjuvant when administered by the sublingual route, which in turn should not comport any risk of retrograde homing to the central nervous system. Unpublished results from our group demonstrated a successful stimulation of robust immune responses following sublingual immunization with formulations containing c-di-AMP, which were comparable to those obtained after i.n. application.

7.6 Mechanism of Th17 differentiation blockage by NKT cells after stimulation with α-galactosylceramide

As mentioned above, Th17 cells have been shown to play an important role in clearance of certain pathogens such as *Candida albicans*, *Klebsiella pneumoniae* or influenza virus [116, 123-125]. However, Th17 cells are also known to be harmful, as they are responsible for autoimmune diseases like RA, MS or IBD [100] and can lead to immune pathological reactions following infection, such as chronic inflammation after *Borrelia* infection [224]. These examples show the importance of controlling the induced Th phenotype when performing vaccinations. Thus, it would be helpful to have substances that can specifically trigger or block Th17 differentiation when added to vaccine formulations. Previous studies showed that immunization by i.n. route always results in strong Th17 responses, which can be either beneficial or unfavorable depending on the pathogen against which has to be vaccinated. This implies problems for this otherwise promising application route. The immunization experiments performed during this work clearly demonstrated that the Th17

phenotype induced by i.n. vaccination can be easily blocked by co-administration of αGCPEG as adjuvant. Furthermore, administration of αGCPEG not only blocks the stimulation of Th17 responses, but also modulates and fine tunes the strength of Th17 induction when combining it with other polarizing adjuvants. The observed blockage of Th17 cells is mediated by NKT cells. This is supported by the results of *in vitro* experiments in which NKT cells stimulated with αGCPEG shut down the generation of IL-17A producing $CD4^+$ T cells, even under Th17 polarizing culture conditions. In addition, real-time PCR analysis revealed that NKT cells stimulated with αGCPEG also inhibit the production of IL-17F and down-regulate the expression of the Th17 specific transcription factor RORγ (data not shown). These results were confirmed by the *in vivo* studies using Jα281 KO mice, which lack functional NKT cells. In these animals an efficient Th17 differentiation was observed even after i.n immunization with αGCPEG as adjuvant. In wild type animals which received the same formulation, a Th17 immune response was absent. Furthermore, *in vitro* studies performed during this work, based on NKT cell co-cultures and the addition of neutralizing Abs, demonstrated that IL-4 and IFNγ released by NKT cells were responsible for the observed Th17 inhibition. Interestingly, the presence of IL-4 or IFNγ alone was sufficient to block the stimulation of Th17 cells. In contrast, simultaneous neutralization of both cytokines fully restored the Th17 response. Thus, the present work revealed for the first time the underlying mechanism of Th17 blockage triggered by αGCPEG. It is remarkable that this blockage mediated by NKT cells is based on the same effects by which Th subsets influence each other's induction. Namely, by the release of IL-4 and IFNγ rather than by any new mediators or cell-to-cell interactions. As this mechanism is already quite well understood, it will allow for a better prediction and targeted investigation of functionality and potential side effects of αGCPEG in order to develop this compound as a vaccine adjuvant.

Another interesting point is the fact that NKT cells not only block the release of IL-17 by inhibiting Th17 differentiation (see also [282]) but also produce IL-17A by themselves [283]. This finding seems to be contradictory to what was reported before, but it is consistent when looking at the chronology of events. In concert with other components of the innate immune system, NKT cells respond very fast after an encounter with a pathogen. Thus, they are an early source of IL-17A which promotes inflammation [284]. It can be hypothesized that later, when the adoptive immune

system starts to respond, NKT cells modulate the Th differentiation to prevent a potentially harmful over-induction of Th17 cells. In this context, a study from Lin *et al.* showed that early IL-17 production is needed to induce Th1 immune responses under certain conditions [285].

The ability of αGCPEG to inhibit Th17 differentiation could be also helpful to investigate if and in which extent Th17 cells are important for protection against certain pathogens. As discussed before (7.5), it is not yet fully clarified if a strong Th17 immune response is beneficial for protection against all influenza strains. Here αGCPEG could be used to selectively block the Th17 differentiation in an immunization and subsequent viral challenge, in a comparative analysis with Th17 inducing adjuvants. An in depth knowledge about the influence of Th17 induction on the outcome of immune responses against pathogens is a crucial step for the improvement and *de novo* development of vaccines.

8 Conclusions and Outlook

The studies performed in this thesis showed that the induction of the Th17 phenotype is not only influenced by the amount of Ag used for stimulation, but also by several other factors, such as the cytokine milieu and co-stimulation. Furthermore, Ag dose dependent Th17 polarization depends on the genetic background of the donor TCR transgenic animals to which the Ag is presented. This is most probably due to differences in TCR affinity to the Ag. In addition, Th17 induction *in vivo* also depends on the adjuvant used and specific dosage of both Ag and adjuvant. Taken together, from the outcome of the experimental work it can be hypothesized that Th17 cells are preferentially induced by low strength stimulation in the absence of polarizing conditions, as well as by strong stimulation in the presence of strong Th17 polarizing conditions.

Intranasal immunization of mice with low dosages of a virosome-based vaccine against the influenza strain H5N1 co-administered with c-di-AMP induced protective immune responses characterized by a strong Th1 and Th17 polarization. Under these experimental conditions the Ag dose needed to elicit protective immunity could be reduced by a factor of 10-15 with respect to previous studies. This represents a considerable dose sparing effect.

It would be of great interest to further investigate the differences observed between the transgenic mouse strains. To this end, a large library of mutated OVA-peptides could be used to investigate the impact of the TCR affinity to the Ag on Th17 polarization exploiting surface plasmon resonance with Biacore technology. It would also be necessary to further validate the results obtained using OVA as Ag by repeating the experiments with influenza virosomes as Ag under less Th17 polarizing conditions. To this end, different application routes or adjuvants need to be included in the immunization protocols.

Finally, for the development of vaccines, not only the induction of Th17 immune responses is of great interest, but also their specific blockage. During this work, secretion of IL-4 and IFNγ by NKT cells was identified to be responsible for blockage of Th17 cell induction by αGCPEG. These findings provide a better characterization

of the underlying mechanisms of adjuvanticity of αGCPEG and highlight its potential to tailor Th17 responses. Additional work will be required to define the true potential of αGCPEG in this regard, such as vaccination and challenge experiments using different infection models (*e.g. Borrelia ssp.*) for which the stimulation of a Th17 immune response represents a drawback for the host.

9 References

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